Universidade de Aveiro Departamento de Química
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Dissertation submitted to the University of Aveiro to meet the requirements for the Degree of Master Biotechnology, performed under the scientific guidance of Prof. Luísa Serafim, Invited Assistant Professor at Department of Chemistry, University of Aveiro, and Dr. Simona Rossetti, Researcher at Istituto di Ricerca sulle Acque – Consiglio Nazionale delle Richerche (IRSA/CNR).

Aos meus Pais…

the jury

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To my Parents and my Sister… Thank you!

palavras-chave

Polihidroxialcanoatos, Culturas Mistas Microbianas, HSSL, Alimentação Aeróbica Dinâmica, FISH, PCR

resumo

A eficácia do processo de produção de polihidroxialcanoatos (PHA) por culturas microbianas mistas (MMC) depende da fase de selecção de culturas. Este passo é determinante para o passo seguinte de acumulação, pois a sua optimização depende da estabilidade e da capacidade de produção da cultura seleccionada.

Neste projecto, uma cultura mista proveniente de uma estação de tratamento de águas residuais, bastante diversificada em termos fenotípicos, foi submetida a condições de alimentação dinâmica aeróbia (ADF), também conhecidas por fome e fartura num reactor descontínuo sequencial (SBR) utilizando como substrato um resíduo da indústria papeleira, *o* licor de cozimento ao sulfito ácido (HSSL). A cultura atingiu um máximo de acumulação de PHA de 67,6 %, verificando-se que dos vários componentes presentes no HSSL, o ácido acético era totalmente consumido, os lenhossulfonatos presentes, parcialmente, e apenas uma pequena pare da xilose era consumida.

Recorrendo-se a *fluorescence in situ hybridization* (FISH) realizou-se a identificação das bactérias acumuladoras de PHA. Foram identificadas bactérias pertencentes aos seguintes grupos: Alfa (72,7 ± 4,0 %), Beta (11,1 ± 0,37 %) e Gamaproteobactérias (10,3 ± 0,3 %). Ao nível do género foi possível identificar, em baixa concentração, *Paracoccus* (4,2 ± 0,51 %) e *Defluvicoccus* (9,0 ± 0,28 %) ambos pertencentes à classe das Alfaproteobactérias. Após plaqueamento da cultura em meio sólido contendo xilose e ácido acético, isolaram-se bactérias identificadas como *Klebsiella spp.* e verificou-se que eram Gamaproteobactérias capazes de acumular PHA a partir de xilose.

keywords Polyhydroxyalkanoates, Mixed Microbial Culture, HSSL, Aerobic Dynamic Feeding, FISH, PCR

abstract

The effectiveness of polyhydroxyalkanoates (PHA) production process includes a first stage of Mixed Microbial Culture (MMC) selection. This step determines the success of subsequent PHA accumulation step performance.

In this project, a MMC collected in a wastewater treatment plant was submitted to Aerobic Dynamic Feeding (ADF) in a Sequencing Batch Reactor (SBR) in order to select PHA-accumulating organisms using hardwood spent sulphite liquor (HSSL), a complex feedstock originated from the pulp industry, as substrate. The selected MMC reached a maximum PHA content of 67.6 %. Acetic acid was fully consumed while, lignosulphonates only partially and only a small part of xylose was taken up.

The selected culture was then analyzed. The identity of PHA-storing bacteria was obtained by fluorescence *in situ* hybridization (FISH). Bacteria belonging to the three main classes were identified: *Alpha*- (72.7 ± 4.0 %), *Beta*- (11.1 ± 0.37 %) and *Gammaproteobacteria* (10.3 ± 0.3 %). At the genus level, only small amounts of *Paracoccus* (4.2 ± 0.51 %) and *Defluvicoccus* related to Tetrad Forming Organisms (9.0 ± 0.28 %) belonging to *Alphaproteobacteria* were detected. After plating the MMC in solid medium containing xylose and acetic acid, colonies of a *Gammaproteobactaria* able to produce PHA from xylose were isolated. Later these colonies were identified as *Klebsiella spp..*

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ABREVIATIONS

3HA	3-hydroxyalkanoate
3HB	3-hydroxybutirate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
ADF	Aerobic dynamic feeding
AN/AE	Anaerobic/Aerobic process
ASTM	American Society for Testing and Materials
C/N	Carbon to nitrogen ratio
CoA	Coenzyme A
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
FISH	Fluorescence *in situ* hybridization
GAO	Glycogen-accumulating organisms
GM	Genetically modified
HAs	Hydroxyalkanoates
HRT	Hydraulic retention time
HSSL	Hard Sulfite Spent Liquor
LS	Lignosulphonates
μ	Specific growth rate
LCA	Life cycle assessment
MAAS	MicroAerophilic-Aerobic System
MCL	Medium chain- length
MMC	Mixed microbial cultures
Mw	Molecular weight
NREL	(American) National Renewable Energy Laboratory
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OLR	Organic loading rate
OUR	Oxygen uptake rate
P(3HB)	Poly(3-hydroxybutyrate)
P(3HV)	Poly(3-hydroxyvalerate)
P(3HB-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

P(4HB)	Poly(4-hydroxybutyrate)
PAO	Polyphosphate-accumulating organisms
PDI	Polydispersity index
PHA	Polyhydroxyalkanoate
PHAs	Polyhydroxyalkanoates
PLA	Polylactic acid
$-q_{Ac}$	Acetic acid specific uptake rate
$-q_{xyl}$	Xylose specific uptake rate
q_{PHA}	PHA specific storage rate
$r_{acetic\ acid}$	Acetic acid uptake rate
r_s	Substrate uptake rate
r_{xylose}	Xylose uptake rate
$r_{ammonium}$	Ammonium uptake rate
RT-PCR	Reverse transcriptase-polymerase chain reaction
RT	Retention time
SBR	Sequencing batch reactor
SCL	Short-chain-length
SRT	Sludge retention time
SSL	Sulfite spent liquor
TCA	Tricarboxylic acid
VFA	Volatile fatty acid
VSS	Volatile suspended solids
WWTP	Waste waters treatment plant
$Y_{PHA/S}$	Yield of PHA on substrate
$Y_{O2/X}$	Yield of oxygen on biomass
$Y_{O2/S}$	Yield of oxygen on substrate
$Y_{X/S}$	Yield of biomass on substrate

1. INTRODUCTION

The increasing interest in new renewable sources of energy and materials is a consequence of several factors. One of these factors is the rapid depletion of fossil fuels reserves that presents a challenging problem to the world, especially to countries heavily reliant on petroleum-based feedstocks. This fast depletion results from the increase in energy consumption by the industrialized nations. Another factor is the exclusive monopoly of oil supply by countries showing political instability resulting in the wavering of petroleum prices. Moreover, the environmental concerns due to the increasing of greenhouse gas emissions from fossil fuels and production of petroleum-based materials constitute another cause for the search of renewable feedstocks (Kamm *et al.* 2006; Sousa *et al.* 2009).

Therefore, a new industrial trend relies on moving from petroleum-based to biomass-based products and sustainable manufacturing processes. The development of a bio-based product industry offers an economical and environmental friendly solution for the surplus agricultural commodities production with low economic income for farmers and for the large amounts of industrial wastes with high disposal (Kamm *et al.* 2006). In this novel bio-based product industry, arose the concept of biorefinery, which was defined by the American National Renewable Energy Laboratory (NREL) as "a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass". Figure 1 shows a schematic representation of the biorefinery concept.

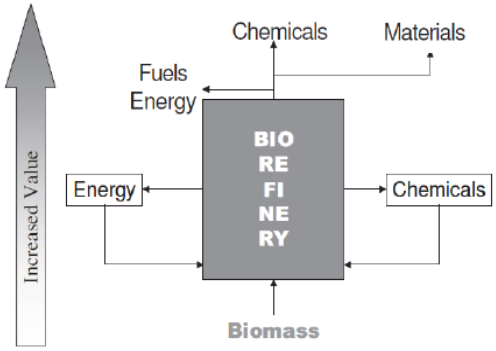


Figure 1: Biorefinary concept (Carvalheiro *et al.* 2008).

When compared to petroleum refineries, biorefinaries can process a wider range of feedstocks using different processing technologies. The productivity and viability of a

biorefinary can be enhanced by integrating the production of higher valuable bioproducts into the fuel and power plants (Carvalheiro *et al.* 2008). It's also possible to enhance the productivity and efficiency resorting to operations that decrease the overall energy intensity of biorefinery's unit operations, once we can integrate and maximize the use of all feedstock components, by-products and waste streams (Yang 2007).

One possible production line which appears from the processes integration is the production of biomaterials, namely the bioplastics, from industrial wastes or by-products (real substrates). The use of conventional plastics raised several problems such as disposable concerns, emissions of greenhouse gases, exhaustion and an increase demand for the fossil resources. In the recent years, bioplastics, more particular polyhydroxyalkanoates (PHAs) produced by mixed microbial cultures arose as an alternative.

In opposition to some bioplastics, already industrially produced, PHAs are completely biodegradable and biocompatible, and can be produced from non-edible raw materials, which are assimilated and converted by the bacterial cultures.

In this way appear this project, which aim is to investigate the production of PHAs by a mixed microbial culture (collected at SIMRia) resorting to a by-product from the pulp and paper industry (Caima S.A.), hardwood spent sulfite liquor (HSSL).

This work intends to study the behavior of microorganism fed with different carbon sources, present in the HSSL, acetic acid, xylose and lignosulphonates. The kinetic and stoichiometric parameters of the culture will be determined with the final goal of optimization of PHAs production under aerobic dynamic feeding (ADF) conditions. Another purpose is to relate the behavior of the selected microbial culture with its evolution along the operational time. Finally, it is intended to identify the best PHA-accumulating microorganism selected by the operational parameters imposed.

The experimental work was divided into three different methodologies, yet all interconnected and complemented to each other. So, the first part is the operation of the sequencing batch reactor under aerobic dynamic feeding from which samples were taken and analyzed. With the selected MMC were made several kinetic batch tests under different conditions. These two parts were accomplished at University of Aveiro during the first semester. Finally, the third phase comprised the microbiological characterization of the SBR biomass and was performed at IRSA-CNR during the second semester.

2. STATE OF THE ART

2.1. BIOPLASTICS

The increasing use of petroleum-based materials in modern lifestyle has raised several concerns. Among these materials, plastics are, undoubtedly, the most important, since they are generally used around the globe for a wide variety of applications, including food packaging, clothes, shelter, communication, transportation or health care (Chen 2009). Due to their wide range of applications, plastics present a serious disposal problem, being the landfills the main system for the disposal of municipal solid wastes. However degradation of plastics occurs with very slow kinetics, hence, the volume required by these materials in landfills is virtually stable over time (Gironi and Piemonte 2011). Following this concerns, biobased and biodegradable polymeric materials may be among the most suitable alternatives for some applications.

Biopolymers or bio-based plastics are polymers that can be synthesized and/or degraded and assimilated by living organisms. The bio-based polymers also include the polymeric materials that are extracted directly from natural resources or are chemically polymerized from bio-based monomers. Besides being biodegradable, many bioplastics are biocompatible since they do not cause any harm to the human body. These characteristic results in a broad range of medical applications (Luengo *et al.* 2003). Currently, there are three major methods for the production of bio-based and biodegradable polymers: a) chemical polymerization of monomers derived from biological process (e.g. NatureWorks® from NatureWorks, U.S. – mostly composed of poly(lactic acid) (PLA)); b) direct biosynthesis of polymers in microorganisms (e.g. Biopol® from Metabolix, U.S. – composed of polyhydroxyalkanoates); c) modification of natural polymers (Mater-Bi®, from Novamont, Italy; and Cellgreen®, from Daicel Chemical Industries, Japan – composed of starch polymers and cellulose derivatives, respectively) (Sudesh and Iwata 2008).

However, not all bio-based polymers are biodegradable, like cellulose ester derivatives or crystalline PLA. According to the American Society for Testing and Materials (ASTM), jointly with the International Organization for Standardization (ISO), the term “biodegradable” means that the material presents the capacity of undergoing

decomposition into CO_2, water, inorganic compounds or biomass by enzymatic action of microorganism.

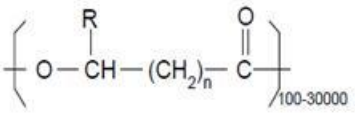
The interest in the development of biodegradable plastics in recent years has both environmental and strategic nature. The conventional plastic production led to the increase of green-house gas emissions and to problems with the disposal of non-biodegradable plastic packaging waste (Gironi and Piemonte 2011). Moreover, the current technology for producing PLA, starch polymers or cellulose derivatives uses raw materials that interfere with human and animal chain food. From these facts, it is obvious that the development and production of bioplastics must rely on a compromise of using less valuable raw materials and more of non-edible raw materials (Kamm *et al.* 2006). Thereby, the development of technologies that use lignocellulosic biomass or wasted crops as feedstocks for the biobased and biodegradable plastics has been emerging in the last years (Sudesh and Iwata 2008).

2.1.1. POLYHYDROXYALKANOATES (PHAS) – HISTORY, CHARACTERISTICS AND PROPERTIES

The occurrence of PHA in bacteria was observed back early last century, when Lemoigne reported the formation of poly(3-hydroxybutyrate) (P3HB) in inclusion bodies of *Bacillus megaterium* (Lemoigne 1926). This discovery was followed by several studies about its function as energy reserve (Dawes and Senior 1973) and the discovery of other polymers beyond P3HB (Wallen and Rohwedder 1974) and new producing species and their potential applications (Anderson and Dawes 1990). However, the high production costs and the availability of low-cost petrochemical-derived plastics set aside bioplastics for a long time (Verlinden *et al.* 2007). Nowadays, the efforts in finding sustainable alternatives inverted this tendency and several PHAs are already being commercialized (Chanprateep 2010). Even so, it is still necessary to continue the research and development to reduce the costs of these bioplastics. The current research is focused in optimized strains, better fermentation processes and the use of inexpensive substrates as reviewed by Verlinden and co-workers. (Verlinden *et al.* 2007).

PHAs are aliphatic polyesters synthesized by living organisms and composed, mostly, by 3-hydroxyalkanotes (3HAs). Due to the stereo-specific enzymes involved in the biosynthesis, the 3HAs monomers present *R* configuration. The high stereoregularity

makes PHAs optically active, and some are highly crystalline. The general chemical structure is presented in Figure 2.



n	R	Full Name
1	hydrogen	Poly(3-hydroxyproprianate)
	Methyl	Poly(3-hydroxybutirate) (P3HB)
	Ethyl	Poly(3-hydroxyvalerate) (P3HV)
	Propyl	Poly(3-hydroxyhexanoate) (P3HHx)
2	Hydrogen	Poly(4-hydroxybutyrate)
3	Hydrogen	Poly(5-hydroxyvalerate)

Figure 2: General chemical structure of PHAs (Reddy *et al.* 2003; Ojumu *et al.* 2004)

The group R (Figure 2) varies from C_1 (methyl) to C_{13} (tridecyl). The carboxyl group of one monomer establishes an ester bond with the hydroxyl group of the neighboring monomer, being catalyzed by a PHA synthase (Verlinden *et al.* 2007). Due to this characteristics, innumerous variations in the length and compositions of side chains are available which allows a large set of potential applications (Verlinden *et al.* 2007).

PHAs can be divided into three groups: the short-chain-length PHAs (scl-PHAs) with 3 to 5 carbons, the medium-chain-length PHAs (mcl-PHAs), which vary between 6 to 14 carbons and the long-chain-length PHAs (lcl-PHAs), with more than 14 carbons (Singh and Mallick 2008). The size of the monomer influences the polymer properties (Yang 2007). For example, the small side chains, methyl and ethyl groups, of scl-PHAs result in a high crystallinity, tensile modulus, and low elongation at break. The large side chains of mcl-PHAs confer elasticity to the material and a relatively low crystallinity and melting temperature, but improved elongation at break (Table 1). Although the most well-studied PHA is P3HB, currently are known more than 150 different hydroxyalkanoates as constituents of these polymers (Dias *et al.* 2006). From these, only P3HB and co-polymers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) are being industrially produced (Dias *et al.* 2006).

Manipulating the process conditions, it is possible to obtain different compositions of PHAs copolymers, allowing for the tailoring of polymers with the desired properties. These strategies aim to approach the tailored materials to the most commercialized conventional plastics like PP, PE and PVC (Lemos *et al.* 2006; Glazer and Nikaido 2007;

Penlogloua *et al.* 2011). In Table 1 are summarized the different physical characteristics of conventional plastics and the most studied PHAs.

Table 1: Physical properties of synthetic plastics vs. typical PHAs (Yang 2007).

Property	PP	LD PE	PS	PVC	P3HB	UP 3HB	P3HB 3HV	P3HB 3HHx	P3HB 4HB
Melting temp. (ºC)	168	123	---*	---*	177	182	140	127	150
Glass temp. (ºC)	-20	-36	90	110	4	4	-1	-1	-7
Cristalinity (%)	60	30	---*	---*	70	80	60	34	45
Young's Modulus (GPa)	1.3	0.4	3.2	3.2	3.5	0.97	0.8	0.5	---*
Tensile Strength (MPa)	36	20	36	46	43	175	20	21	26
Elongation break (%)	350	530	2	60	5	104	50	400	444
Notched Izod impact strength (J/m)	50	NB**	24	580	60	---*	110	---*	---*

Note: *not available; **no break.

PHAs are stored intracellularly as discrete granules in inclusions bodies, of 0.2–0.5 μm in diameter, as shown in Figure 3. Once the polyesters are synthesized, they can serve as carbon and energy sources during starvation periods. The PHA content in most bacteria, however, is low, ranging, normally, from 1 to 30 wt % of cell mass. Suspended in cytoplasm, the inclusions bodies have, approximately, 5 to 10 wt % of water and are largely amorphous (Sudesh and Doi 2000 ; Yang 2007). Each granule is surrounded by a phospholipid monolayer membrane in which proteins, including PHA synthase and degradase, are located (Mccool and Cannon 1999). Other proteins (phasins) are presumed to be involved in the stabilization of the amorphous hydrophobic PHA inside the hydrophilic cell cytoplasm (Fuller 1999).

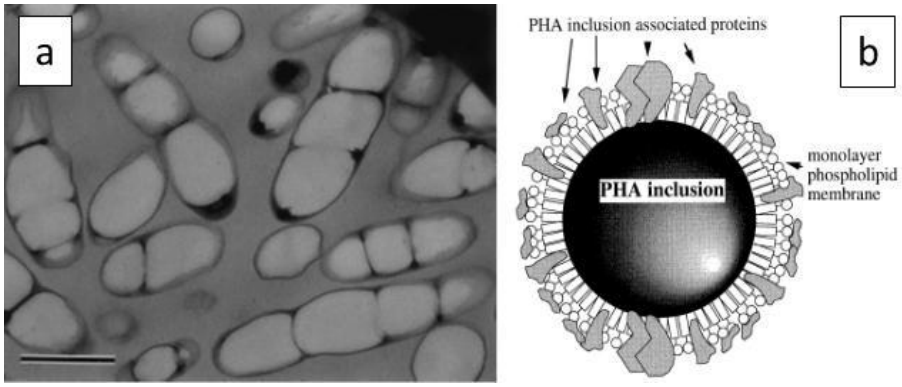


Figure 3: a)Transmission electron micrograph of thin sections of recombinant *R. eutropha* with inclusions bodies with PHB; b) A model attempting to show the structure of in vivo PHA inclusions (Sudesh and Doi 2000).

Another important property of PHAs is their complete biodegradability in a natural environment (Figure 4) that sets PHAs apart from the conventional plastics, and even from others bioplastics (Khanna and Srivastava 2005; Sudesh and Iwata 2008). The degradation of PHA starts with a hydrolysis step, in which, a random non-enzymatic chain scission of ester groups, leading to reduction in molecular weight. The degradation is affected by the rate of diffusion of water through the polymer. The biodegradation process of bioplastics depends on both environmental factors (temperature, moisture, oxygen, pH) and the chemical structure of the polymer. These polymers contain ester hydrolysable bonds in the polymer backbone with increased susceptibility to biodegradation. Other factors that affect biodegradability rely on crystallinity, molecular weight, and, in the case of copolymers, the monomeric composition (Sudesh and Doi 2000 ; Verlinden *et al.* 2007).

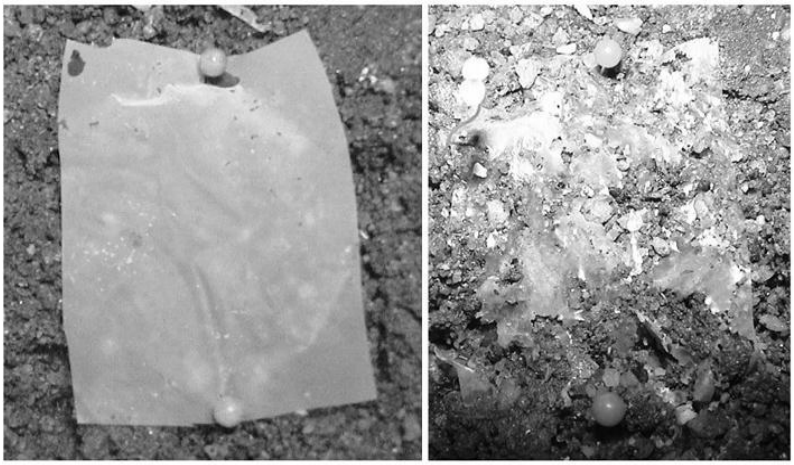


Figure 4: Degradation of PHA film, in 50 days, placed on soil surfaces under tropical conditions, during 50 days (Sudesh and Iwata 2008).

Jendrossek and Handrick claimed that the ability to degrade extracellular PHA is widely distributed among bacteria and fungi, through aerobic and/or anaerobic metabolism, in soil, compost, aerobic or anaerobic sewage sludge, fresh and marine water, estuarine sediments and even in air (Sudesh and Doi 2000 ; Jendrossek and Handrick 2002). This capability is dependent on specific extracellular enzymes (e-PHA depolymerases) that are able to degrade the crystallized PHA into water soluble oligomers and monomers, and therefore the microorganisms utilize the resulting products as nutrients. These enzymes are not necessarily synthesized by PHA accumulating microorganisms, which further increases the possibility of degradation of PHAs (Jendrossek and Handrick 2002; Choi *et al.* 2004). Finally, since biological production of PHA can be based on renewable resources (like agro-forestry), their fully biodegradability allows for the complete fulfilling of the natural carbon cycle, as shown in Figure 5.

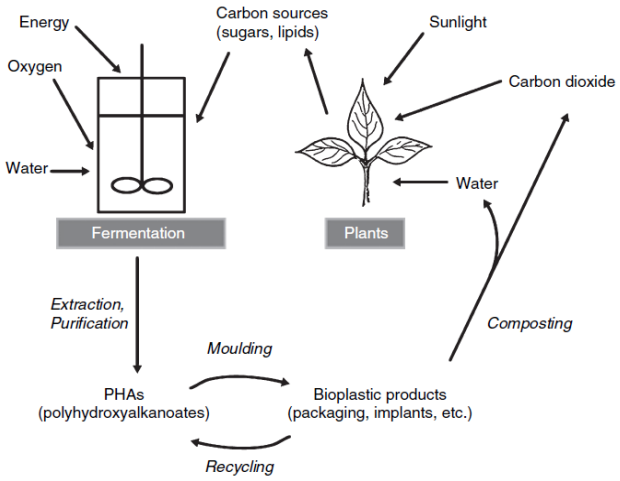


Figure 5: Life cycles of PHAs (Gross and Kalra 2002).

2.2. APPLICATION OF POLYHYDROXYALKANOATES

Over the last decade, applications of PHAs have increased both in variety and specialization. In recent years, the number of research publications dealing with biosynthesis, fermentation, and characterization of the PHA family of biopolymers has increased (Philip *et al.* 2007). The most usual applications of bioplastics are as disposable items but also as biocompatible medical devices (Philip *et al.* 2007). PHAs applications can be divided into three major areas: industrial, agricultural and medical. Due to their diverse structures and properties, PHAs have penetrated different markets sectors as

bioplastics, fibers, biomedical implants and drug delivery carriers, among others as presented in Table 2.

Table 2: Application of PHA in various fields (Chen 2009).

Applications	Examples
Packing industries	All packing materials that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances, etc.
Printing & photographic industry	PHAs are polyesters that can be easily stained.
Other bulk chemicals	Heat sensitive adhesives, latex, and smart gels. PHA nonwoven matrices can be used to remove facial oils.
Block copolymerization	PHA can be changed into PHA diols for block copolymerization with other polymers.
Plastic Processing	PHA can be used as processing aids for plastic processing.
Textile Industries	Like nylons, PHA can be processed into fibers.
Fine chemical industries	PHA monomers are all chiral *R-forms*, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals.
Medical implant biomaterials	PHAs have biodegradable and biocompatibility, and can be developed into medical implant materials. PHA can also be turned into drug controlled release matrices.
Medical	PHA monomers especially R3HB, have therapeutic effects on Alzheimer's and Parkinson's diseases, osteoporosis and even memory improvement.
Healthy food additives	PHA oligomers can be used as a food supplements for obtaining ketone bodies
Industrial microbiology	The PHA synthesis operon can be used as a metabolic regulator or resistance enhancer to improve the performances of industrial microbial strains
Biofuels or fuel additives	PHA can be hydrolyzed from hydroxyl-alkanoate methyl esters that are combustible.
Protein purification	PHA granules binding proteins phasin or PhaP are used to purify recombinant proteins.
Specific drug delivery	Coexpression of PhaP and specific ligands can help achieve specific targeting diseased tissues.

2.2.1. INDUSTRIAL APPLICATIONS

Industry, in generally, is one of the areas where the use of plastics assumes major proportions (Sudesh and Iwata 2008) and so, the introduction of bioplastics are of extremely importance. Even though bioplastics industry is still identifying and exploring different market sectors (Queiroz and Collares-Queiroz 2009), the use of bioplastics in some industries has been remarkable (Philip *et al.* 2007). Bioplastics, nowadays, are being used for the manufacture of short-lived materials and products like films, catering products, food packing, packing and waste bags, paper coating (Bioplastics 2011; Coats *et*

al. 2011). PHAs are also used for durable products, such as mobile phone covers, cars and electronic components, flushable sanitary/household product backing materials (Sudesh and Iwata 2008; Queiroz and Collares-Queiroz 2009). Due to the piezoelectric nature of PHAs, items like pressure sensors, material testing, shock wave sensors or gas lighters can be also produced (Philip *et al.* 2007).

2.2.2. MEDICAL APPLICATIONS

Due to their biocompatibility PHAs have been gaining ground in the field of medical applications and reaching, progressively, the most innovative biomedical fields (Rodríguez-Carmona and Villaverde 2010). Based on their characteristics, PHAs have been used mostly in drug delivery, tissue engineering, wound dressing in surgery, wound management, bioimaging, and biosensors with great success (Yao *et al.* 2008; Atwood and Rehm 2009; Wu *et al.* 2009). Among others, P(3HB), P(3HB-3HV), Poly(4-Hydroxyvalerate) (P(4HV)), Poly(3-hydroxyoctanoate) (P(3HO)) and Poly(3-Hydroxybutyrate-co-3-Hydroxyhexanoate) (P(3HB-3HHx)) have been used frequently in tissue engineering (Philip *et al.* 2007) as well as in nerve conduit, carriers and scaffolds (Wu *et al.* 2009).

2.2.3. AGRICULTURAL APPLICATIONS

Regarding to this area, the principal uses of PHAs in agriculture is in the controlled release of fertilizers, herbicides and insecticides (Philip *et al.* 2007). Some commercial products that allow for this control are already in use. Nodax^{TM}, a copolymer of 3HB and 3HHx was tested as coating for fertilizers and herbicides; in 1985, Holmes and co-workers used P(3HB-3HV) in the controlled release of insecticides as the polymer was degraded by the insects (Holmes 1985).

Another application is as bacterial inoculants to enhance nitrogen fixation in plants. Therefore, these inoculants improve the survival and development of the vegetable species, and for that the storage of PHA by bacteria must be high to bear adverse conditions of the environment (Kadouri *et al.* 2003; Kadouri *et al.* 2005).

More agricultural applications include encapsulation of seeds, biodegradable plastic films for crops protection and biodegradable containers for hot house facilities (Verlinden *et al.* 2007).

2.3. POLITICAL/ECONOMICAL ANALYSIS AND LIFE CYCLE ASSESSMENT

Nowadays, the assessment of the impact of a new industrial process on the economics and on the ecosphere is an obligatory prerequisite for a sustainable industrial development (Koller *et al.* 2011). This has been verified due to policies imposed around the world that incentive the development and implementation of new processes and materials (such as bioplastics, namely PHAs). These polices also restrict the materials that do not present any sustainable advantage over those already implemented (Queiroz and Collares-Queiroz 2009; Chanprateep 2010; Koller *et al.* 2011). As examples, in Europe, countries like Ireland, Scotland, Denmark and Sweden have already imposed levies and taxes on non-degradable plastics bags. Also USA, the San Francisco Board of Supervisors approved "first-in-the nation" legislation that outlaws the use of non-biodegradable plastics bags in large supermarkets within 6 months and large chain pharmacies in about 1 year. Also Asia, Japan and India created laws on promoting green purchasing and recycling and banned the use of plastics in some regions (Chanprateep 2010).

Despite the incentive polices, the economic role is the core of all development and investment of sustainable materials. Thereby, environmental degradable polymers, like PHA, must be economically competitive with petroleum-based plastics. At the beginning of industrial production of PHAs, the prices hovered 10 – 12 €/kg, which was not competitive with conventional plastics nor with other bio-based polyesters (e.g. starch polymer) (Chanprateep 2010). However, the main reason for the high prices was mainly owing to the raw materials used like carbon source and the energy spent on maintenance on pure culture, between 40 to 60 % (Akiyama *et al.* 2003; Gurieff and Lant 2007; Chanprateep 2010). Nevertheless, with the evolution of technology, like genetic engineering, optimization of operational conditions or the improvement of the monitoring techniques for the control of microbial growth, lower prices were obtained that allowed for enlargement of the market quota of PHAs. Table 3 shows some cases of currently commercial polymers based on PHA and their market prices in 2010.

Table 3: Commercial PHA, manufactures and market prices in 2010 (Chanprateep 2010).

Polymer	Trade names	Manufactures	Capacity (tons)	Price (kg^{-1})
PHB	Biogreen®	Mitsubishi Gas Chemical Company (Germany)	10000	€2,5-3,0
PHB	Mirel™	Telles (US)	50000	€1,50
PHB	Biocycle®	PHB Industrial Company (Brazil)	50	n/a
PHBV and PHB	Biomer®	Biomer Inc. (Germany)	50	€3,0-5,0
PHBV, HBV+ Ecofelx blend	Enmat®	Tianan Biologic Ningbo (China)	10000	€3,26
PHBH	Nodax™	P&G(US)	20000-50000	€2,50
PHBH	Nodax™	Lianyi Biotech (China)	2000	€3,70
PHBH	Kaneka PHBH	Kaneka Corporation	1000	n/a
P(3HB-co-4HB)	Green Bio	Tianjin Gree Bio-Science (Japan)	10000	n/a
PHA from P&G	Meredian	Meredian (US)	272000 (2013)	n/a

The continuous research on this area and the investment in the development of new technologies along with the high acceptance from general population and pressure applied by several governments, have led to a growing market, all over the world, for the biopolymers (Figure 6).

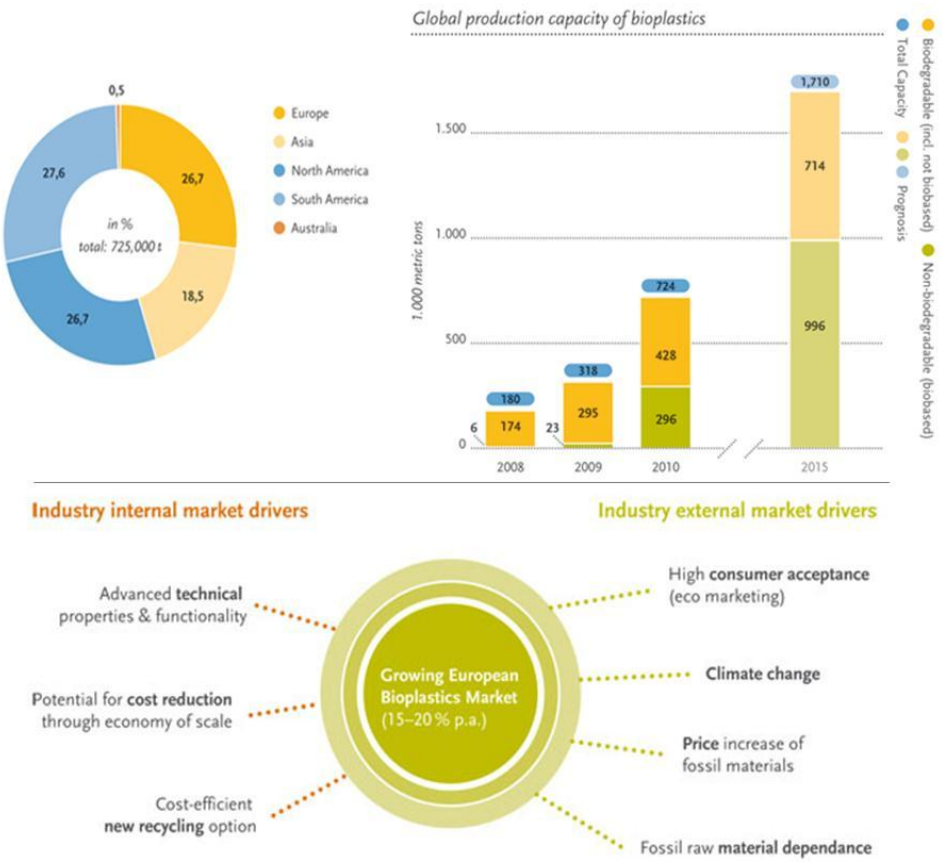


Figure 6: Markets drivers and tendencies (Bioplastics 2011).

With the continuous demand for cheaper methodologies for PHAs production, mixed microbial cultures (MMCs) and renewable raw materials/renewable waste residues have been the subject of several scientific studies (Serafim *et al.* 2004; Serafim *et al.* 2008; Albuquerque *et al.* 2010; Arcos-Hernandez *et al.* 2010; Villano *et al.* 2010; Moita and Lemos 2011). MMCs had attracted attention regarding, primarily, to its potential to produce significant amounts of PHAs with apparently lower costs owing to lower sterility needs, equipment and control requirements and the capacity to use a wide range of cheap substrates, namely wastes from industry and municipalities (Coats *et al.* 2011) and agro-forestry wastes (Reis *et al.* 2003; Bengtsson *et al.* 2008; Castilho *et al.* 2009; Albuquerque *et al.* 2010; Morgan-Sagastume *et al.* 2010). However, this approach must compete economically with other polymers production processes and with treatment processes for the same waste resource (Gurieff and Lant 2007). A Life Cycle Assessment (LCA) allows for the analysis of the process sustainability and determines its economic viability compared to processes already established (Koller *et al.* 2011). Figure 7 schematizes the LCA process tools.

Notwithstanding its importance, there are only a few works about LCA of PHAs production processes. Relating to mixed culture, Gurieff and Lant (2007) asserted that the entire production process of PHA by MMCs needs an optimization but, none the less, there are already many benefits such as the carbon neutral feedstock (no need for pure substrates derived from food crops) and no necessity of sterile conditions. These advantages lead to a more sustainable process than those with pure cultures or the classical process for the production of petroleum-based plastics.

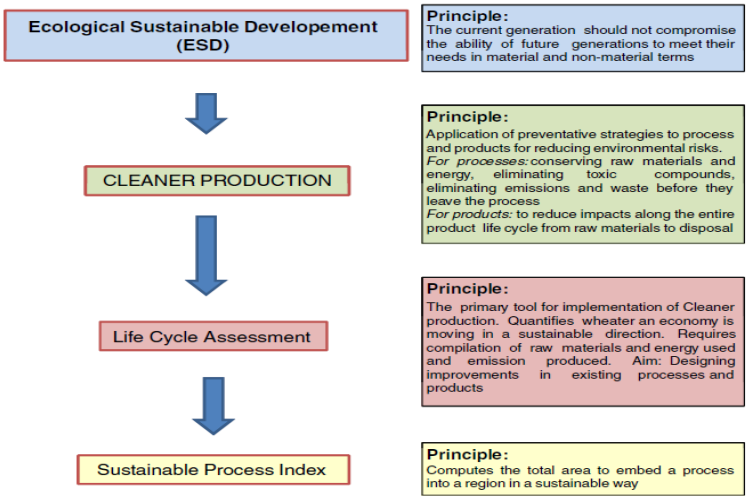


Figure 7: LCA's tools (Koller *et al.* 2011).

Hereupon, this work aims the optimization and selection of the best storage PHA population selected from a mixed culture using an industrial waste as carbon source, meeting the objective of a sustainable and profitable process.

2.4. PRODUCTION OF PHAS

In the recent years, numerous approaches were studied for the production of PHAs, making use of a variety of organisms such as bacteria, plants and fungi (Khanna and Srivastava 2005). Also, with the advent of genetic engineering, the use of several recombinant organism has become possible, like the well-known recombinant *Escherichia coli* or new transgenic plants (Koller *et al.* 2011). With the aim of achieving the industrial production there are several considerations to take into account: choosing cheap and suitable substrates in accordance with the metabolism of the producing organism; its storage capacity; the need for sterile conditions; type of reactor, feeding pattern; oxygen demand; behavior and evolution of the culture (in case of a MMC), among others (Coats *et al.* 2007; Serafim *et al.* 2008).

Over than 300 bacterial species were reported as PHAs producers but only 75 were tested for large-scale production. Currently, industrial production process settles on pure cultures of microorganism in their wild form, and recombinant microorganisms, like *Ralstonia eutropha*, *Alcaligenes latus*, *Aeromonas hydrophila*, *Bacillus spp.*, *Pseudomonas putida*, *Pseudomonas oleovorans* and *Escherichia coli* (Reis *et al.* 2003; Salehizadeh and Van Loosdrecht 2004; Dias *et al.* 2006; Chen 2009; Akaraonye *et al.* 2010). As already mentioned, genetic engineering allowed for the construction of recombinant strains for a much more cost-effective PHAs production. By cloning PHA synthesis genes from several organisms it was possible to obtain simultaneously fast growth and high cell density with the ability to use inexpensive substrates and simple polymer purification. However, the use of pure culture and genetically modified organisms still presents several drawbacks and one way to overcome them is the use of mixed microbial culture (MMC).

2.4.1. PURE CULTURES VERSUS MIXED MICROBIAL CULTURE

The main cost drivers of PHAs production relies in the cost of the substrate used by pure cultures (Choi and Lee 1997) and energy for sterilization (Serafim *et al.* 2008).

Moreover, the PHA content strongly affects the efficiency of the recovery process, and consequently the overall cost (Serafim *et al.* 2008).

MMCs have emerged as an alternative to pure cultures once operating costs can be reduced by 50 %. Additionally, with the use of raw materials such as agro-industrial waste costs operating costs can go down to 85 % (Salehizadeh and Van Loosdrecht 2004; Albuquerque *et al.* 2010). The use of MMCs and low value substrates can also result in energy saving, since there is no need for sterilization. The equipment costs can be also minimized due to less process control required (Serafim *et al.* 2008).

Activated sludge from wastewater treatment plants (WWTPs), a well-known MMC can be an illustrative example since it is able to store PHA as carbon and energy source under transient conditions. The microorganisms developed in WWTPs usually experience frequent changes in the availability of nutrients and have the capacity to adapt continuously to it. These microorganisms are able to quickly store the available substrate and consume it during periods of lack of nutrients, which is a strong competitive advantage over the organisms without this capacity (Coats *et al.* 2007; Serafim *et al.* 2008).

When compared with pure cultures, which maximum production ascend to more than 90 wt % as reviewed by Chen in 2009, with the MMC has already been registered a PHA content of 88 wt % in a 8 hours period with synthetic medium (Johnson *et al.* 2009) and 74,6 wt % obtained with real medium, such as fermented molasses (Albuquerque *et al.* 2010)). With these results and future improvements in the technology, the advantages of PHA production in open mixed culture would be an enhanced economy, a simpler process control, no requirement of monoseptic processing and an improved use of wastes.

2.4.2. MECHANISM AND METABOLISM FOR PHAS PRODUCTION BY MIXED MICROBIAL CULTURES

MMCs are defined as unknown microbial populations capable of intra- and extracellular specific reactions, selected by imposed conditions on the microbial community. The production of PHAs by these cultures was first verified in biological phosphorus removal process, where under alternating conditions of anerobiosis and aerobiosis (AN/AE), two main types of microorganism: polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO) compete for the available carbon substrate (Serafim *et al.* 2008). As explained by Serafim and collaborators (2008),

in these two groups, the PHAs synthesis plays an important role in their metabolism. Under anaerobic conditions, the carbon source is used for the production of PHAs with simultaneous consumption of glycogen and, under aerobic conditions PAOs and GAOs consume the stored PHA for growth, maintenance and glycogen replenishment.

Other possible configuration for PHA production using MMCs is the aerobic dynamic feeding (ADF) process. In this process, periods of excess of carbon alternate with substrate limitation, favoring the selection of the PHAs-accumulating communities. The absence of external substrates for considerable periods of time leads to a decreased of intracellular components necessary for cell growth (like RNA and several enzymes). After this famine phase, if an excess of carbon is supplied, due to the low availability of material essential for cell growth, some microorganisms accumulate it as PHAs (Serafim *et al.* 2008; Liu *et al.* 2011).

The metabolism for PHA synthesis is well established for pure cultures, but only few works report metabolic studies for MMCs (Lemos *et al.* 2006; Lemos *et al.* 2007). It is possible to assume that PHA metabolism in MMCs is similar to that described for pure cultures (Dias *et al.* 2006), which is shown in Figure 8. In PHAs production metabolism, the three main enzymes, β-ketothiolase, acetoacetyl-CoA NADPH-dependent reductase and PHB synthase, are codified by the structural genes phbA, phbB and phbC (Figure 8b). These enzymes are located in the cell cytoplasm, where the accumulation of reserve polymer granules under the appropriated conditions takes place (Luengo *et al.* 2003).

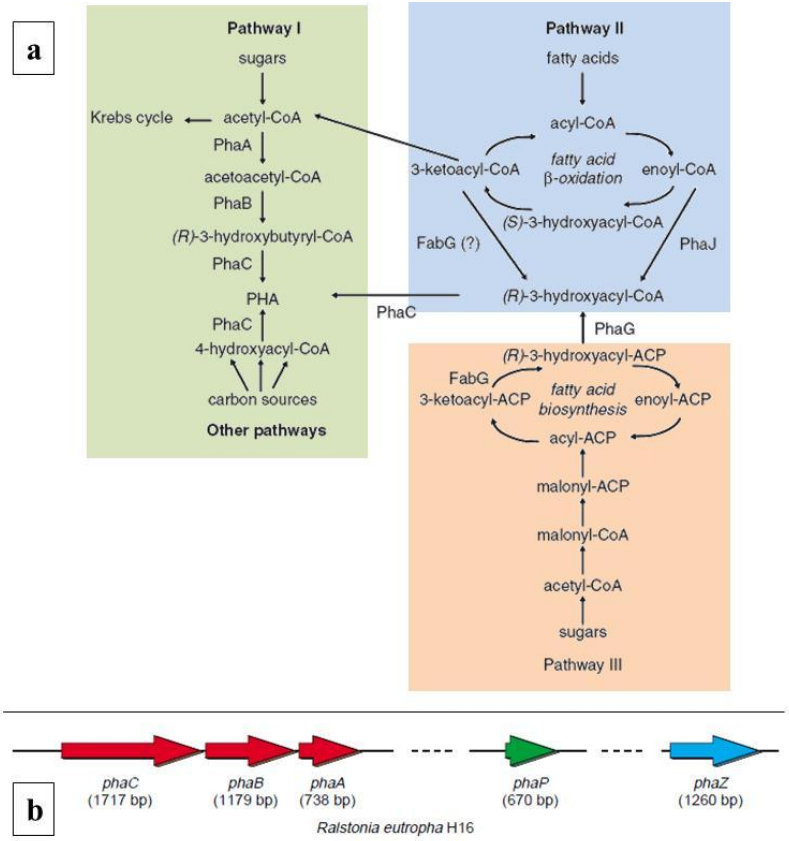


Figure 8: a) Metabolic pathways supplying monomers for PHAs synthesis (Tsuge 2002); b) Organization of the main genes involved in the biosynthesis PHAs of *Ralstonia eutropha* (Luengo *et al.* 2003)

The key intermediate compound in the synthesis and degradation of PHAs is the acetyl-CoA. During the consumption of external substrate, the production of acetyl-CoA occurs, being partially drifted to the production of PHAs and growth through the citric acid cycle (TCA). The synthesis of PHB, in *R. eutropha*, is based on three enzymatic reactions catalyzed by enzymes mentioned above (Figure 8a, green zone).The first reaction is the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA and catalyzed by the β-ketothiolase, encoded by phbA. The second reaction is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase, encoded by phbB. Finally, the monomers of (*R*)-3-hydroxybutyryl-CoA are polymerized with the intervention of the PHB synthase enzyme, encoded by phbC (Luengo *et al.* 2003). This last enzyme is also responsible for the polymer chain length and formation of an enzyme-oligomer complex, from which begins the micelle formation (Sudesh and Doi 2000).

Usually, the feedstock supplied to PHAs-producing organisms is enriched in organic acids (normally a mixture of VFAs for MMC) and/or sugar-based compounds,

common in pure cultures, which follow different metabolic pathways. Moreover, the overall polymer composition reflects the combination of the different substrates. For example, and assuming that PHA metabolism in MMCs, as previous referenced, is similar to that described for pure cultures, in MMC the organic acids are transported across the cell membrane and activated to acyl-CoA (Figure 8a, blue zone). In the case of acetate, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, by β-ketothiolase, and then, reduced to 3-hydroxybutyryl-CoA, by acetoacetyl-CoA reductase, with simultaneous conversion of NADPH to $NADP^+$. 3-hydroxybutyryl-CoA is the precursor used by PHB synthase to the production of P(3HB) (Serafim *et al.* 2008). Regarding to propionate, two molecules of propionyl-CoA condense and they originate 3-hydroxy-2-methylvaleryl-CoA, the precursor for poly-3-hydroxy-2-methylvalerate, P(3H2MV). If acetyl-CoA is present or formed from the breakdown of propionyl-CoA, the junction of both molecules, acetyl and propionyl-CoA, can produce poly-3-hydroxyvalerate, P(3HV), or poly-3-hydroxy-2-methylbutyrate, P(3H2MB) (Serafim *et al.* 2008). When propionate is the sole carbon source, acetyl-CoA can be obtained through citric acid pathway. Despite 3-hydroxyvalerate production was supposed to be the main pathway for propionate transformation, some 3HB formation was observed since 3-hydroxybutyryl-CoA, the precursor of this units, resulted from the condensation of two acetyl-CoA units (Lemos *et al.* 2006). Other VFAs, including, butyrate and valerate, can lead to the formation of scl-PHA and mcl-PHA (Pisco *et al.* 2009).

2.5. PROCESS OPERATION

PHAs synthesis by MMCs has been widely studied in the last years as reviewed by Serafim *et al.* (2008). Such studies were focused on the methodologies for the selection of PHA storing cultures, increasing the amount of PHA accumulated, reactors operational strategies and on different substrate usage, which can be included in the two/three-stage process. The two-stage process includes selection of PHA-accumulating organisms, which is the aim of this project, followed by PHA accumulation, where the PHA storage, by the selected culture, is maximized. In the three-stage process, there is a pre-fermentation step that is required to transform carbohydrates into VFAs and other organic acids, further used in the selection and accumulation steps (Dionisi *et al.* 2005; Serafim *et al.* 2008).

2.5.1. CULTURE SELECTION

The objective of this step is to select the maximum number of organisms from the initial culture, with a high PHA storage capacity. In this process, the system is operated in order to eliminate the microorganisms presenting low or no storage capacity since they contribute for the reduction of the average PHA cell content. This is a crucial step once very heterogeneous populations resulting in low overall storage capacity could have a negative impact in the downstream process. However, keeping a high storage capacity leads to an unstable population in the selection reactor. Thus, the operation of the selection reactor should be optimized to obtain a homogeneous population with relatively high and stable storage capacity rather than to maximize the PHA cell content (Serafim *et al.* 2008). In here, two main processes can be highlighted, AN/AE and ADF.

The AN/AE process allows for the selection of two main groups of microorganisms, PAOs and GAOs, which, under anaerobic conditions, use the carbon source for the production of PHAs with simultaneous consumption of glycogen. Under aerobic conditions, after the exhaustion of the external substrate, the stored PHA is consumed for growth, maintenance and replenishment of glycogen reserves (Serafim *et al.* 2008). GAOs present a higher robustness under these conditions, when the main objective is the PHA production (Dai *et al.* 2007). It should be noted that, this process, was already tested with enriched real substrates like fermented wastewater with 53 % in PHA (w/w) (Coats *et al.* 2007), pre-fermented paper mill effluent that potentiated the PHA accumulation up to 48 % (w/w) (Bengtsson *et al.* 2008) and fermented sugar cane molasses (Pisco *et al.* 2009).

The MicroAerophilic-Aerobic System (MAAS) was introduced by Satoh and collaborators (1998) and resulted from a modification of the AN/AE process: a limited amount of oxygen is supplied during the anaerobic period (Satoh *et al.* 1998; Takabatake *et al.* 2000). In this system, microorganisms consume organic substrates that allow for the achievement of energy through oxidative degradation. If the supply of oxygen is sufficient, the microorganisms are able to get enough energy for assimilative activities such as the production of protein, glycogen, along with consuming of organic substrates. Based on this, Reis and co-workers (2003) asserted that if the supply of oxygen is adequately controlled, the assimilative activity will be suppressed while giving a way to the microorganisms to accumulate PHAs (Reis *et al.* 2003).

Finally and with regard to ADF, the most studied PHA storage process by MMCs, the mechanism of accumulation was first proposed in 1996 by Majone *et al.* (1996). In this process, also known as feast and famine, the MMC, under aerobic conditions, submitted to alternated periods of external substrate availability (feast) and unavailability (famine) results in unbalanced growth. As mentioned before, the absence of a carbon source during a period of time causes a decrease in the amount of intracellular components fundamental for the cell growth (RNA and enzymes). Following this period of famine, if the MMC is fed with an excess of carbon, as the amount of enzymes available is lower than that necessary for a maximum growth rate, the storage of PHAs became the dominant phenomena (Majone *et al.* 1996). As stated in the previous section, VFAs are the mainly carbon source used by MMC to synthesis PHAs. Under ADF conditions, Reis and co-workers (2003) have proposed a possible metabolic pathway for acetate consumption, as illustrated by Figure 9.

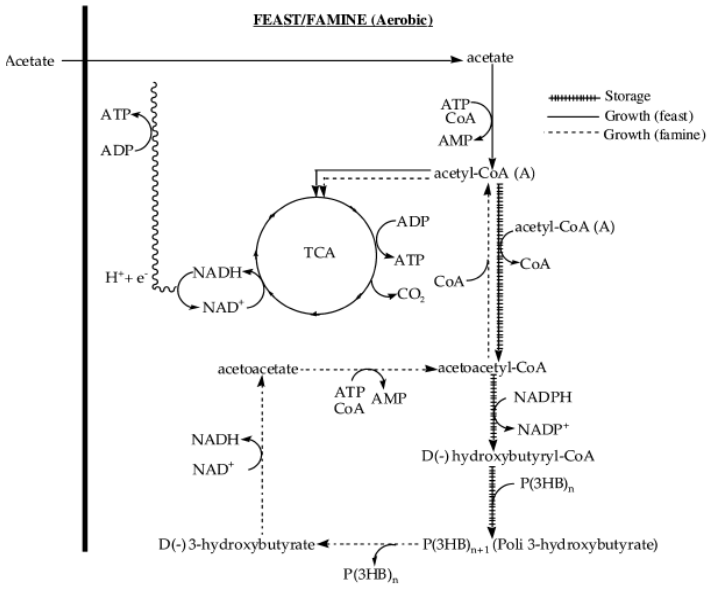


Figure 9: Possible metabolic pathway for acetate consumption under ADF conditions by MMC (Reis *et al.* 2003)

As reviewed by Serafim and co-workers (2008), the selection of MMCs under ADF conditions can be performed using real complex wastes such as fermented cane molasses, fermented palm oil mill effluents, industrial wastewaters, fermented paper mill wastewater, tomato cannery waste or fermented brewery wastewater.

To complement the selection of the PHA-storing populations, the evolution of microbial community must be evaluated in order to identify the best producers and

determine the individual relative abundance. This correlation would allow for the design of operating conditions favoring the most important PHA-accumulating microorganisms (Serafim *et al.* 2008).

2.5.1.1. MICROBIAL COMMUNITY ANALYSIS

To develop a most competitive PHA production process using MMC, in order to monetize the further production process, the selection of microorganisms' stage, with a high storage capacity, must take place. Moreover throughout the system operating period, the population must be checked in order to understand some changes in the performance of MMCs that eventually occurred. For these reasons, it is necessary to monitor the population evolution, in order to identify the different groups of microorganisms and relate them with the storage capacity and kinetics of the MMC.

In the few studies found in the literature, molecular approaches were applied to identify the PHA accumulating organisms selected under ADF conditions (Dionisi *et al.* 2005; Dionisi *et al.* 2006; Serafim *et al.* 2006; Lemos *et al.* 2008; Moita and Lemos 2011). Resorting to DGGE and 16S rRNA clone library, Dionisi and co-workers, in 2005 and 2006, followed the evolution of a PHA-storing MMC and identified three main families, *Betaproteobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* (Dionisi *et al.* 2005; Dionisi *et al.* 2006). In 2006, Serafim *et al.* with Fluorescence *In Situ* Hybridization (FISH) corroborated the results from Dionisi, verifying that *Betaproteobacteria* and *Alphaproteobacteria* where present in their culture as main PHA producers (Serafim *et al.* 2006). Later in 2008, Lemos *et al*. (2008), using sorting/Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) revealed the main PHA accumulating organisms (at genus level) in a system fed with different substrates, acetate and propionate, that allow the selection of different PHA accumulating organism. Resorting to this method, the authors were able of selectively separate bacteria with a distinctive morphology from the microbial community by micromanipulation and then the collected cells were utilized for successive 16S rRNA gene amplification by RT-PCR. They also used FISH to estimate the relative abundance of the microorganisms identified by sorting/RT-PCR (Lemos *et al.* 2008). Bacteria belonging to the genus *Amaricoccus*, *Thauera* and *Azoarcus* were identified, but with differences in their relative abundance that could be explained due to the different VFAs used (Lemos *et al.* 2008; Jiang *et al.* 2011a). In other cultures studied,

Plasticicumulans acidivorans and *Thauera selenatis* were identified as the predominant bacterial species, which used lactate as carbon source (Jiang *et al.* 2011a). Besides the type of substrates used, the temperature also has influence on the microbial community selection, being identified *Zoogloea* and *Plasticicumulans acidivorans* as the main PHAs accumulators (Jiang *et al.* 2010).

2.5.2. REACTOR OPERATIONAL STRATEGY

Most of the works that studied the production of PHAs by MMC submitted to ADF conditions were carried out in sequenced batch reactors (SBRs), operated with cycles of feeding, reaction, settling and drawn (Dias *et al.* 2006). In these reactors, the selective pressure that favors PHA-accumulating microorganisms is maintained during the reactor operation. Dionisi and co-workers (2005) adopted a three stage process that consist, firstly, a pre-fermentation step, where carbohydrates are converted to a mixture of suitable VFAs, followed by the selection step in a SBR operated under ADF conditions, and finally, a stage where a batch reactor is fed with a high substrate concentration to increase the polymer content (Dionisi *et al.* 2005; Albuquerque *et al.* 2007). Following this methodology described by Dionisi *et al.* (2005), the ideal configuration would be a small SBR for the enrichment of microorganism with a high cell growth and PHA accumulation, from which the inoculum for a large reactor is removed. In here, with a different strategy, it would promote the cell growth followed by PHAs production with cell growth limitation. In this, there are four main parameters which can influence the yield of PHA storage to take into consideration: nitrogen concentration, pH, oxygen concentration and temperature (Dias *et al.* 2006).

Regarding to the pH control, several studies were reviewed by Dias *et al.* (2006) finding that pH values between 6 – 7 led to a lower PHA content than those with pH in the range of values 8 – 9. Besides that, in a recent study, Villano *et al.* (2010) concluded that the pH control allowed for a controlled production of co-polymer P(3HB/3HV) (Villano *et al.* 2010). Sometimes, some conflicting results between different studies may be due to different MMC used, and with different levels of selection, that not always behave in the same manner.

About dissolved oxygen concentration (DO), according to Third *et al.* (2002), when oxygen is present in limiting concentrations it can lead to higher yields of PHA storage.

This can be explained by the limited availability of ATP at low DO concentrations, that prevents significant biomass growth, since it is used to transport the substrate into the cell where is converted to reserve material (Third *et al.* 2003).

The temperature can also influence PHA accumulation, since at higher temperatures (35 ºC) a decrease in the yield of PHB was observed by Krishna and Van Loosdrecht (1999). This could be an advantage because low temperature allows for a less costly process as it influences the production of PHA (Krishna and Van Loosdrecht 1999). However, Johnson and co-workers founded that at 30 ºC occurred the maximum production of PHB, and with the temperature decrease the production of PHB has also declined (Johnson *et al.* 2010). These apparently contradictory results could be explained by the use of different mixed microbial cultures inocula, which may have undergone by a previous adaptation to the reactor at different temperature (Johnson *et al.* 2010), or even due to the different feeding strategy applied by these authors (Jiang *et al.* 2010).

2.5.3. SUBSTRATE

The substrate cost is the one of the main contributors to the high cost of PHA production. It has been estimated that 40 – 50 % of the total PHA production cost is due to the type of substrate used (Dias *et al.* 2006). For this reason, a more cost-effective process should include the selection and preparation of cheap substrates, which can be successfully used by microorganism to synthetize PHA at high productivities. Also the substrate must be converted into a polymer with the suitable properties that fit in a wide range of industrial applications. In the past years, a wide variety of low-cost carbon substrates, already mentioned, have been used for the production of PHAs by MMC (Coats *et al.* 2007; Bengtsson *et al.* 2008; Albuquerque *et al.* 2010).

The choice of the substrate should, wherever possible, meet the concept of the biorefinery. Substrates like wastes from several industries (food, agricultural, forest, paper) must integrate production plants that comprise suitable technologies for their conversion into valuable intermediate and final products, fuels and energy (Kamm *et al.* 2006). Among the different types of biorefinaries, a lignocellulosic-based biorefinery could be the most successful one because of the abundance and affordability of raw materials, wide variety and good marketing of the bio-based products. Following this, the substrate chosen to be used in this project was the Hardwood Sulphite Spent Liquor (HSSL), originated

from the pulping of *Eucalyptus globulus* wood during the paper production process. The wood from eucalypt suffers a mechanical and chemical treatment as outlined in Figure 10.

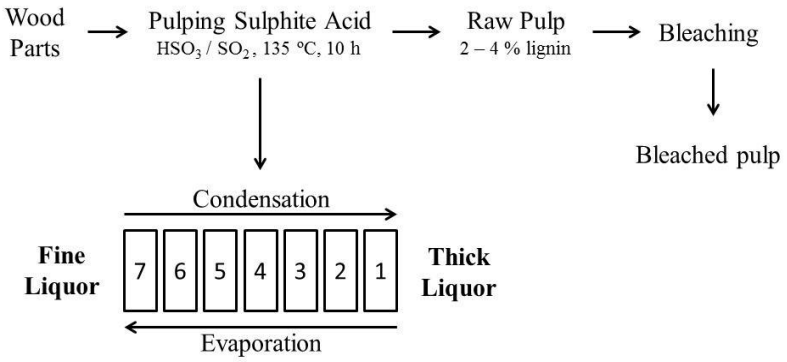


Figure 10: Pulp and paper process by the use of sulfite acid and Mg^{2+} base. The SSL used in this work is a waste product from the process and is collected from the 7th evaporator. Adapted (Pontes 2008).

The HSSL is a side product from sulphite pulp production and, besides sulphonated lignin, contains sugars from degraded hemicelluloses, mainly pentoses (Table 4). Using of HSSL as raw material to produce added value products fits well to the biorefinery concept, in order to decrease the dependence from fossil resources and to improve the economic sustainability of pulp mills (Xavier *et al.* 2010). An advantage of HSSL, comparing to agro-forestry residues, lies on lignocellulosic fraction that has already suffered a hydrolysis step releasing most of the monomeric sugars, xylose, mannose and arabinose.

Table 4: Composition of eucalypt sulphite spent liquor (Xavier *et al.* 2010).

Components	Concentration (g/L)
Lignosulphonates	78.2 ± 0.6
Acetic Acid	8.2 ± 0.3
Furfural	< 0.1
Ash	19.8 ± 0.2
D-Xylose	24.6 ± 0.5
D-Mannose	8.5 ± 0.9
L-Arabinose	7.8 ± 0.3
D-Galactose	4.5 ± 0.1
D-Glucose	2.3 ± 0.1
L-Rhamnose	1.6 ± 0.3
L-Fucose	0.4 ± 0.3

Studies performed under ADF conditions showed that the microorganisms from MMC consume, preferentially, the VFAs, like acetic acid (Reis *et al.* 2003). For this

reason, HSSL is a good candidate as a renewable, inexpensive and sustainable substrate for PHA production by MMC, since it contains a significant amount of acetic acid, 8.2 g/L. If the selected MMC does not consume the sugars, they can be used for a subsequent production of second-generation bioethanol, as proposed by (Xavier *et al.* 2010).

3. METHODS AND MATERIALS

3.1. CULTURE

The mixed microbial culture used in this worked was collected from wastewater treatment plant Aveiro North (SIMRia 2011).

3.2. BIOREACTOR

In this study, a sequenced batch reactor (SBR) was operated for 10 weeks, Figure 11, in order to select a stable PHA-storing population. The reactor working volume was 1.5 L and the SBR was operated under aerobic dynamic substrate feeding, during which alternating feast and famine phases were imposed per cycle. Each cycle length was 12 hours, comprising 10.5 hours of aerobiosis, 1 hour of settling (with agitation and aeration switched off), 0.5 hours of withdrawing (half of the reactor volume was removed by a a Watson – Marlow Pump 101 F/R) and finally, the volume replacement with fresh medium for period of 15 minutes (with Watson – Marlow SCI 400 pump). From this, a hydraulic retention time (HRT) of 1 day was obtained. The biomass concentration in the bioreactor was kept between 1.5 – 2.0 g/L by purging 150 mL at end of the aerobic period, resulting in a sludge retention time (SRT) of 5 days. Reactor stirring (300 rpm), aeration and feeding and withdrawing pumps were controlled with timers. Dissolved oxygen and temperature were measured with Oxygen meter Transmitter M300 (Mettler-Toledo Thornton, Inc). The system worked without pH control, but its values were monitored (Crison-PH 28 P).

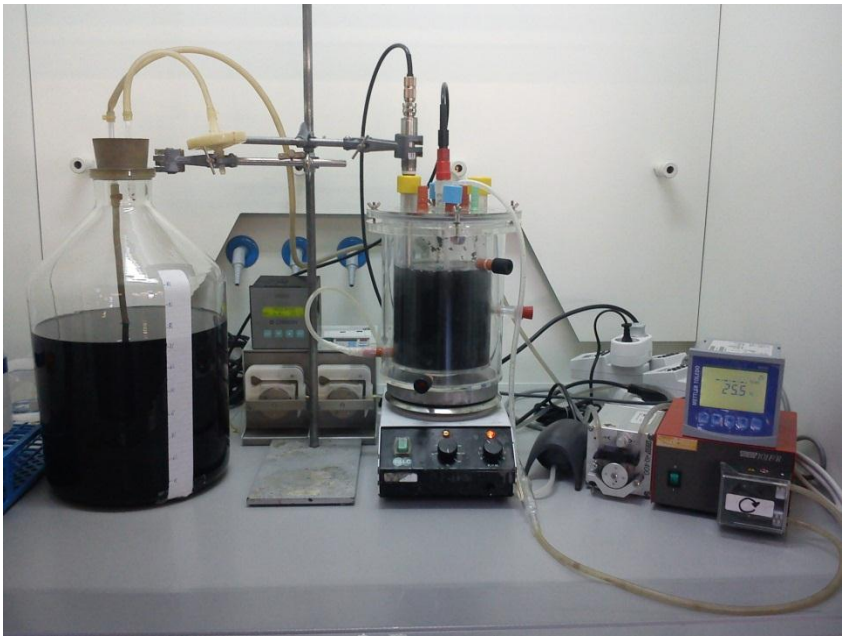


Figure 11: The bioreactor and feeding; aeration pump and feeding and withdrawing pumps; oxygen/temperature and pH meters situated in a laboratory fume hood.

3.3. CULTURE MEDIUM

HSSL pretreatment: The hardwood spent sulphite liquor (HSSL) from magnesium based acidic sulphite pulping of *Eucalyptus globulus* was supplied by *Caima – Indústria de Celulose SA* (Constância, Portugal). Pre-evaporated HSSL was collected from inlet evaporator in a set of multiple-effect evaporators to avoid the presence of free SO_2 in liquor. The pretreatment of HSSL consisted in pH adjustment to 7.0 with 6M KOH followed by aeration with compressed air (2 $h.L^{-1}$). Then, the liquor was centrifuged for 1 hour at 2000 rpm. The precipitated colloids were filtered off using a 1 μm glass microfiber filter (Ahlstrom, grade 131) (Xavier *et al.* 2010).

Medium composition: Culture medium composition is listed in Table 5. Phosphate salts were prepared apart, in order to avoid an irreversible precipitate with magnesium salts during sterilization. To inhibit nitrification, 4 g of thiourea were added. The flasks with the medium and carbon source were sterilized in autoclave. The pH of the feeding, before sterilization, was kept above 6.50 and the phosphates were further added to the flask in a laminar flow hood.

Table 5: Micronutrients concentration and Carbon source.

Compound	Concentration (g/L)
KH_2PO_4	0.016
K_2HPO_4	0.064
$MgSO_4 \cdot 7H_2O$	0.160
$CaSO_4 \cdot 2H_2O$	0.080
$FeCl_3$	0.020
$Na_2MoO_4 \cdot 2H_2O$	0.008
NH_4Cl	0.160
CH_4N_2S	0.4
HSSL	15 mL/L

3.4. SAMPLING

The collection of samples for analysis were made at the beginning of the feeding (t = 0 min), immediately after the stop of feeding pump (t = 15 min) and then at intervals of 1 hour during the cycle. The pH, temperature and percentage of dissolved oxygen in the bioreactor were recorded at the time of the sample collection. The samples were further centrifuged at 14000 rpm for 10 minutes (Mettich Zentrifugen MIKRO 120) and after separating the solid and the supernatant, the supernatant pH was measured (Hanna Instruments, HI 9321 Microprocessor, pH meter), and both stored in the freezer under -16 °C. The supernatant was used to measure the acetic acid and xylose, ammonium and lignosulphonates consumption, by HPLC, Thermo Scientific Ion Selective Electrode and UV Spectrophotometer, respectively. The pellet was used to PHA determination.

3.5. KINETIC TESTS

Several tests were carried out in a reactor with a working volume of 0.5 L without temperature control and sterile conditions. The respirometer was coupled to the bioreactor by silicon tubing and the circulation of the medium was provided by a peristaltic pump (Gilson Miniplus 3), with a controlled flow of 75 cm^3/min. The stirring in the reactor and the respirometer was kept constant, 400 rpm, and only the reactor had a supply of compressed air controlled by a flowmeter at 90 cm^3/min, as shown in Figure 12.

In each test 250 mL of inoculum was used after withdrawn from the main bioreactor before the feeding period. After collecting the sample, every 30 minutes, the percentage of dissolved oxygen was recorded each 5 seconds for 3 minutes.

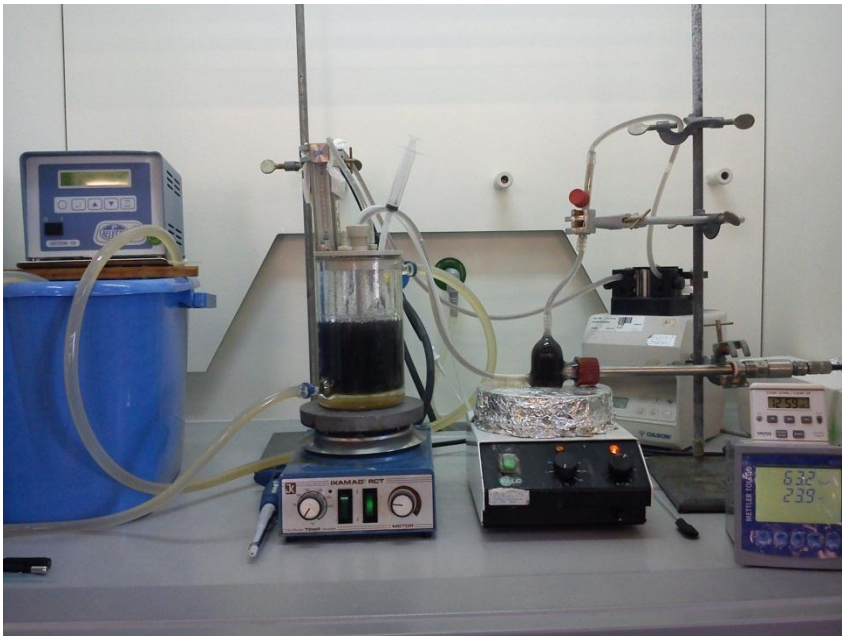


Figure 12: Reactor, respirometer and oxygen meter for the kinetic tests.

Six tests were performed with this methodology regarding to the carbon source: the first three comprise the evaluation of the feeding used in the main bioreactor; five pulses of feeding; and finally normal feeding without ammonium. The feeding had the same composition and it was in same proportion as the main reactor, with exception of the third test. In the last three kinetics tests were used only acetic acid, only xylose and a mixture of both, as carbon sources for each test respectively. The amount of each carbon source used was calculated according to the corresponding COD in the liquor. At the end of the test with the mixture of acetic acid and xylose, the PHA was extracted. The biomass was placed in a small flask with chloroform, under agitation, 180 rpm, during 4 days at 28 ºC. The cellular content was removed and chloroform evaporated.

3.6. Analytical Methods

3.6.1. Carbon Source Analysis

Acetic acid and xylose were measured by HPLC in order to determine the evolution of concentration of acetic acid and xylose in the samples taken. 400 µL of the samples were filtered using a membrane of 0.2 µm (Whatman) at 10000 rpm (Mini Spin Eppendorf) for 15 minutes, and injected (auto sampler – HITACHI L2200) in an ion exchange column Eurokat® of 10 mm, connected to a pump (HITACHI L2130) and refractive index detector

HITACHI L2490. The column temperature was 40 °C (Oven Gecko 2000) and the eluent was H_2SO_4 0.01 N at a flow rate of 0.4 mL/min at room temperature. A calibration using a standard curve for acetic acid and xylose was applied (Xavier *et al.* 2010).

3.6.2. CELL DRY WEIGHT

The amount of biomass was accounted as cell dry weight. 5 mL of sample were filtrated using previously dried and weighted filters (Cellulose Acetate Filter, 0.2 µm pore size, Sartorius) with vacuum filtration. The membranes were placed in the oven at 105 °C for 72 hours. After cooling down they were weighted and the biomass concentration was determined in g/L of suspended solids (SS)

3.6.3. PHA QUANTIFICATION

This quantification was done according with the methodology described by Yilmaz *et al.* (2005) with some modifications. The pellets obtained after the centrifugation of samples were suspended in 1 mL of water. 2 mL of 2 M HCl were added to the suspension and heated at 100 °C for 4 hours in a dry bath. The solution was then left to precipitate, the liquid phase was poured away and 5 mL of chloroform were added to the resulting precipitate. The tubes were left overnight at 28 °C on a shaker at 150 rpm. Then 2 mL of solution was dried in the fume hood with N_2. 5 mL of 2 M sulphuric acid was added. The tubes were heated at 100 °C in a water bath for 2 hours. After cooling to 25 °C, the amount of PHB was determined on a UV Spectrophotometer (Shimadzu UVmini-1240) at 235 nm (Yilmaz *et al.* 2005). The calibration was done resorting to a standard curve.

3.6.4. AMMONIUM QUANTIFICATION

The ammonium concentration was followed using a Thermo Scientific Ion Selective Electrode. To 1 mL of the samples was added 20 µL of Ionic Strength Adjuster (ISA). This solution is composed of 5 M NaOH, 0.05 M EDTA, 10 % methanol and provides a constant background ionic strength and adjusts the solution pH. The calibration was done resorting to a standard curve. Each concentration was measured during 5 minutes. The samples values were recorded in mV, after measurement time of 5 minutes.

3.6.5. LIGNOSULPHONATES QUANTIFICATION

The control of lignosulphonates (LS) content was performed according to Restolho *et al.* (2009). The measurement was performed using a UV Spectrophotometer (Shimadzu UVmini-1240) at 273 nm, after a dilution of 1:80 (Restolho *et al.* 2009). The lignosulphonates concentration was calculated resorting to the Beer-Lambert law, using a $\varepsilon = 7.41\ g^{-1}cm^{-1}$ (Xavier *et al.* 2010).

3.7. MICROBIAL COMMUNITY ANALYSIS

The biomass was harvested by centrifugation and ressuspended in 1xPBS. This wash was performed 4 more times. The formaldehyde (4 %) was then added to the eppendorf with biomass ressuspended in 1xPBS, and then incubated in 4 ºC for 2 hours. The fixed sample was centrifuged (5 min, 4 °C, 15.000 g) and the supernatant poured away. To the ressuspended sample in 1 volume of ice-cold 1xPBS, 1 volume of ice-cold 96 % (v/v) ethanol was added. The sample was stored at -20 °C (Amann *et al.* 1990).

3.7.1. GRAM STAINING

After sample fixation on slide, followed by air drying, the microscope slide was stained for 1 minute with crystal violet solution. Then the solution was removed with water. Further, the slide was treated with solution of lugol for 1 minute and then washed out with water, decolorized with acetone and dried. Lastly, the slide was covered with a solution of safranin for 1 minute and then washed with water. The slide was left to air dry and then it was observed under oil immersion at 1000x magnification with direct illumination resorting to Zeis Axioskop equipped with JVC TK-128OE Color Video Camera (VLC software), Figure 13 (Jenkins *et al.* 1986).

Figure 13: Optic microscope Zeis Axioskop equipped with JVC TK-128OE Color Video Camera

3.7.2. NEISSER STAINING

Thin smears were prepared on microscope slides. After being thoroughly air dried, the slide was covered with methylene blue plus crystal violet solution for 30 seconds, followed by the removal of the solution with water. Additional the slide was treated with bismark brown solution for 1 minute and then washed out with water. The slide was left to air dry prior its observation under oil immersion at 1000x magnification with direct illumination, Figure 13.

3.7.3. NILE BLUE STAINING

Thin smears were prepared on microscope slides. After drying, the slides were submerged in a nile blue solution (1 % w/v), pre-heated at 55 ºC, for 10 minutes. The slides were then recovered and washed with an 8 % acetic acid solutions for 1.5 minutes. The slide was left to air dry and then it was observed under oil immersion at 1000x magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 14 (Rees *et al.* 1992).

Figure 14: Epifluorescence microscope Olympus BX51, equipped with an Olympus XM10 camera

3.7.4. FISH ANALYSIS

Before FISH analysis, in order to decrease the cellular aggregation of the fixed biomass, small aliquots were transferred to new eppendorfs with glass beads, with a diameter of 1mm. These eppendrofs were taken to vortex for 60 seconds (Ghosh 2006).

5 µL of biomass fixed on formaldehyde (4 % v/v) were placed in individual wells of a Teflon-coated slides and then air dried. The slide was then dehydrated in ethanol series (3 minutes each) 50 %, 80 % and 98 %. 10 µL of hybridization buffer (previously prepared and according to stringency of the probe(s) used) were added to each well on the slide, the remainder was used to moisten a tissue paper for the hybridization chamber, and finally 0.5 µL of probe (50 ng/µL) were added to the wells. The slide was placed in the hybridization chamber at 46 ºC for 1.5 hour. After, the slide was lightly washed with the pre-heated washing buffer, and then placed in a Falcon tube, containing the washing buffer, for 15 minutes at 48 ºC. Cold Milli-Q water was used to remove the wash buffer from the slide. After air-drying, slides were mounted with Vectorshield mounting medium containing DAPI stain. (Amann *et al.* 1995). The list of the probes used is shown in the Table 6.

The slide was observed under oil immersion at 1000x magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 14.

With the objective to enhance the signal, a pre-treatment was applied. Cells were first treated with 2500 units of lysozyme (Sigma, 50000 U/mg) in a Tris-EDTA buffer (0.1 mol/L Tris, 0.05 mol/L EDTA), pH 8, for 10 minutes at room temperature and then dehydrated sequentially in 50, 80 and 98 % ethanol for 3 minutes. Thereafter, the cells were exposed to 50 units of mutanolysin (Sigma, 12258 U/mg) in 0.1 mol/L phosphate buffer, pH 6.8, for 10 minutes at room temperature, also followed by the dehydration step (Maszenan *et al.* 2000).

Table 6: Probes and their sequences used in FISH.

Probe	Sequence (5' – 3')	Target	References
EUB338	GCTGCCTCCCGTAGGAGT		
EUB338 II	GCAGCCACCCGTAGGTGT	Bacteria	(Amann *et al.* 1995)
EUB338 III	GCTGCCACCCGTAGGTGT		
Delta495a	AGTTAGCCGGTGCTTCTT		
Delta495b	AGTTAGCCGGCGCTTCCT	Deltaproteobacteria	(Loy *et al.* 2002; Lücker *et al.* 2007)
Delta495c	AATTAGCCGGTGCTTCCT		
Lgc354a	TGGAAGATTCCCTACTGC		
Lgc354b	CGGAAGATTCCCTACTGC	Firmicutes (Gram$^+$ bacteria with low GC content)	(Meier *et al.* 1999)
Lgc354c	CCGAAGATTCCCTACTGC		
Gnsb941	AAACCACACGCTCCGCT	Chloroflexi (green nonsulfur bacteria)	(Gich *et al.* 2001)
Alf968	GGTAAGGTTCTGCGCGTT	Alphaproteobacteria (except Rickettsiales)	(Neef 1997)
Bet42a	GCCTTCCCACTTCGTTT	Betaproteobacteria	(Manz *et al.* 1992)
Gam2a	GCCTTCCCACATCGTTT	Gammaproteobacteria	(Manz *et al.* 1992)
Hgc69a	TATAGTTACCACCGCCGT	Actinobacteria (high GC Gram$^+$ bacteria)	(Roller *et al.* 1994)
Pla46	GACTTGCATGCCTAATCC	Planctomycetales	(Neef *et al.* 1998)
Cf319a	TGGTCCGTGTCTCAGTAC	Flavobacteria, Bacteroidetes, Sphingobacteria	(Manz *et al.* 1996)
Arc915	GTGCTCCCCCGCCAATTCCT	Archaea	(Stahl and Amann 1991)
TM7905	CCGTCAATTCCTTTATGTTTTA	Candidate division TM7	(Hugenholz *et al.* 2000)
DF988*	GATACGACGCCCATGTCAAGGG	Defluvicoccus	(Meyer *et al.* 2006)
DF1020*	CCGGCCGAACCGACTCCC		
TFO-DF218	GAAGCCTTTGCCCCTCAG	Defluvicoccus related Tetrad Forming Organism	(Wong *et al.* 2004)
TFO-DF618	GCCTCACTTGTCTAACCG		
SBR9-1a	AAGCGCAAGTTCCCAGGTTG	Sphingomonas	(Beer *et al.* 2004)
THAU646	TCTGCCGTACTCTAGCCTT	Thauera sp.	(Lajoie *et al.* 2000)
AZO644	GCCGTACTCTAGCCGTGC	Azoarcus sp.	(Hess *et al.* 1997)
PAR651	ACCTCTCTCGAACTCCAG	Paracoccus	(Neef *et al.* 1996)
AMAR839	CCGAACGGCAAGCCACAGCGTC	Amaricoccus spp.	(Maszenan *et al.* 2000)

* required the use of unlabelled helper probes.

3.7.5. BACTERIAL ISOLATION

Another step in this work comprised the attempt of the isolation of some PHA accumulators. For that objective a synthetic mineral, salts and vitamins medium (MSV) (Table 7) were prepared with the major carbon sources (Sodium acetate (CH_3COONa) – 0.172 g/L and Xylose ($C_5H_{10}O_5$) – 0.360 g/L) present in the HSSL. The amount of agar used was of 15 g/L. The solution was then autoclaved and left to cool down in a laminar flow wood. After cooling, it was added the vitamin mix (1 mL/L) to the medium, which was then distributed to petri dishes.

Table 7: Synthetic medium – Micronutrients and Vitamins concentration.

	Compound	Conc. (g/L)
Vitamins	Biotin	0.020
	Folic Acid	0.020
	Pyridoxine	0.100
	Thamine	0.050
	Riboflavin	0.050
	Nicotinic Acid	0.050
	Pantothenic Acid	0.050
	Vitamin B_{12}	0.002
	4-Aminobenzoic Acid	0.050

	Compound	Conc. (g/L)
Micronutrients	KH_2PO_4	0.085
	K_2HPO_4	0.110
	$MgSO_4 \cdot 7H_2O$	0.100
	$CaCl_2 \cdot 2H_2O$	0.080
	$FeCl_3 \cdot H_2O$	0.002
	Na·EDTA	0.003
	$(NH_4)_2SO_4$	0.500

50 µl of the sludge from the reactor was spread onto agar growth medium and the inoculated plates were therefore incubated at 20 °C. Pure culture of a PHA storing Gammaproteobacterium was isolated by cycles of replating onto MVS plus xylose agar plates. A pure culture of the isolate, called DS1, is maintained in slant culture at 4 °C. The purity was evaluated performing gram coloration and FISH analysis.

The culture was then inoculated in liquid medium, Erlenmeyer of 50 mL with MSV and xylose, to determine the growth rate. Then, a 400 mL Erlenmeyer was inoculated with an inoculum from the previous liquid medium. Samples were taken to evaluate the culture growth (absorbance at 420 nm), xylose consumption (use of dinitrosalicylic acid reagent (Miller 1959)) and to perform Nile Blue staining.

3.7.6. PCR

This approach was applied on the isolate obtained. For this, a TaKaRa Ex Taq^{TM} kit was utilized. Four eppendorfs were prepared with general reaction mixture as described by the kit. The primers used were 27 forward (f) and 1492 reverse (r), Table 8. For the PCR reaction was used the Perkin Elmer GeneAmp PCR Sytem 2400 programed with the following cycles:

- 36 ºC for 5 minutes (1 cycle);
- 48 ºC for 5 minutes (1 cycle);
- 48 ºC for 1 minute, 72 ºC for 2 minutes and 34 ºC (28 cycles);
- 48 ºC for 1 minute and 72 ºC for 5 minutes (1cycle).

After the last cycle, the samples were cooled down until 4 ºC and an agarose (1 %) electrophoresis was performed to check if there was amplification.

The samples were then send to BioFabre (Rome, Italy) for sequencing, using the following primers 530f, 926f, 907r and 519r, Table 8. After receiving the results, the sequences were assembled and run into BLAST.

Table 8: Primers and its sequences

	Primers	Sequence (5' – 3')
PCR	27f	AGAGTTTGATCMTGGCTCAG
	1492r	TACGGYTACCTTGTTACGACTT
Sequencing	530f	GTGCCAGCMGCCGCCG
	926f	AAACTYAAAKGAATTGACGG
	907r	CCGTCAATTCMTTTRAGTTT
	519r	GWATTACCGCGGCKGCTG

M = C:A; Y = C:T; K = G:T; R = A:G; W = A:T; all 1:1

3.8. CALCULATION OF KINETIC AND STOICHIOMETRIC PARAMETERS

The sludge PHA content was calculated as a percentage of SS on a mass basis:

$$\%\ \mathrm{PHA} = \frac{\mathrm{PHA}}{\mathrm{SS}} \times 100\ (\mathrm{gPHA/gVSS})$$

where SS includes active biomass (X) and PHA. Active biomass was calculated by subtracting PHA from VSS. The maximum specific substrate uptake ($-q_S$ in gS/gX.h) and PHA storage rates (qPHA/gX.h) were determined by adjusting a linear function to the experimental data of substrate (acetic acid and xylose) and PHA concentrations plotted over time, calculating the first derivative at time zero (taking the slope of the fitting) and dividing the value thus obtained by the active biomass concentration at that point. The yields of PHA ($Y_{PHA/S}$ in gPHA/gS) and active biomass ($Y_{X/S}$ in gX/gS) on substrate consumed were calculated by dividing the amount of PHA formed or the active biomass formed by the total amount of substrate consumed, respectively. The respiration yield on substrate ($Y_{O2/S}$ in gO2/gS) was calculated by dividing the decreased amount of O_2 consumed by respiration (in g/L) per amount of substrate consumed (in gS/L). Since no nitrification occurred, because thiurea was added to the feeding medium, and based on the premise that the molecular formula for biomass is $C_5H_7NO_2$, it was possible to establish that for the production 8 mg of active biomass there is a need of 1 mg of N (Serafim *et al.* 2004). This relation allowed for the calculation of the specific growth rate of the active biomass. The cell dry weight was quantified as volatile suspended solids (VSS) technique according to standard methods (in the work VSS is presented as biomass). The specific growth rate was calculated resorting to the following formula:

$$\mu = \frac{1}{\mathrm{X}} \times \frac{d\mathrm{X}}{d\mathrm{t}}$$

4. RESULTS AND DISCUSSION

4.1. SEQUENCING BATCH REACTOR

4.1.1. SBR PERFORMANCE DURING THE OPERATIONAL PERIOD

The SBR was operated for 67 days and along the operational period several parameters were followed: the uptake of acetic acid and xylose (the main carbon sources present in the HSSL) and the production PHA and biomass. Since HSSL is a complex mixture of carbon sources, the total carbon oxygen demand (COD) should be measured but on that time that was not possible due to some technical problems.

Figure 15 presents the evolution of acetic acid and xylose uptake rates, the two of the major carbon sources of HSSL. The results show a clear preference for acetic acid than for xylose since the MMC assimilated the acetic acid at higher rates, (values in the range 0.098 ± 0.02 g/L.h) than xylose (0.026 ± 0.006 g/L.h) during the entire operation period. Although MMCs are known to preferentially consume VFAs for PHA production, xylose uptake was not a surprise since some sugars, like glucose were reported to be consumed for glycogen production (Carta *et al.* 2001) and Liu *et al.* (2008) reported the use of tomato cannery wastewater for PHA production, a raw material rich in carbohydrates (Prior and Potgieter 1981; Liu *et al.* 2008).

Although it is not shown, it is important to mention that during some cycles the xylose concentration increased occasionally. This increase could be explained by the xylose presence in HSSL: ca. 70 % is in the form of monomeric sugar and remaining 30 % correspond to xylo-oligosaccharides (XOS) (Xavier *et al.* 2010). Under specific conditions this XOS could be degraded increasing the xylose concentration, since some of microorganisms usually present in MMC possess the enzymes needed for the hydrolysis of oligosaccharides (Muralikrishna *et al.* 2011).

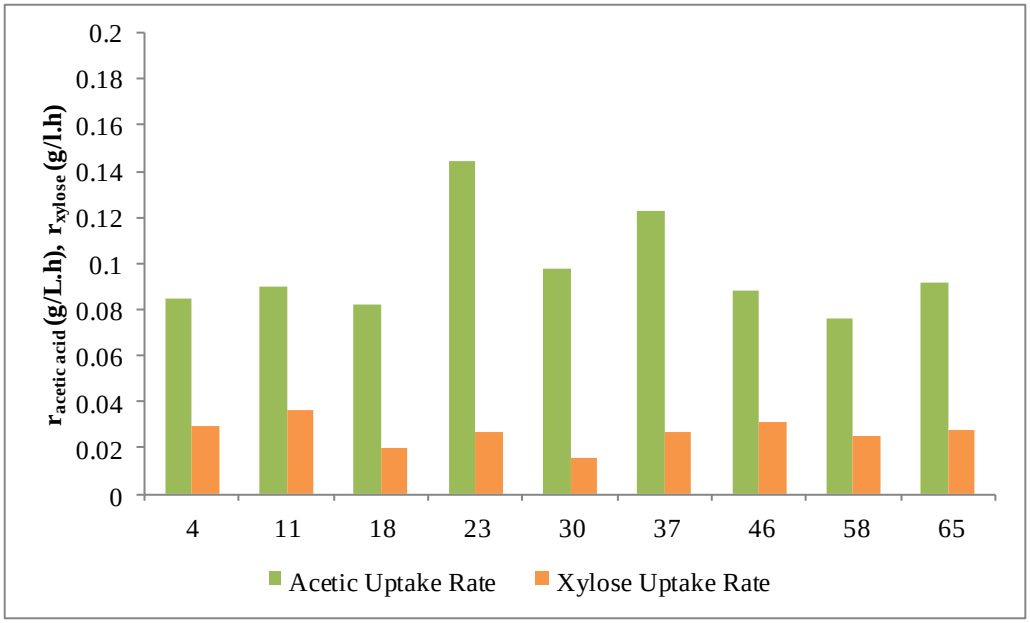


Figure 15: Acetic Acid and Xylose uptake rates during the operational period of the SBR.

Figures 16 and 17 present the PHA and Active Biomass concentration, the specific growth rate and the ammonium uptake rate obtained along the SBR operational period.

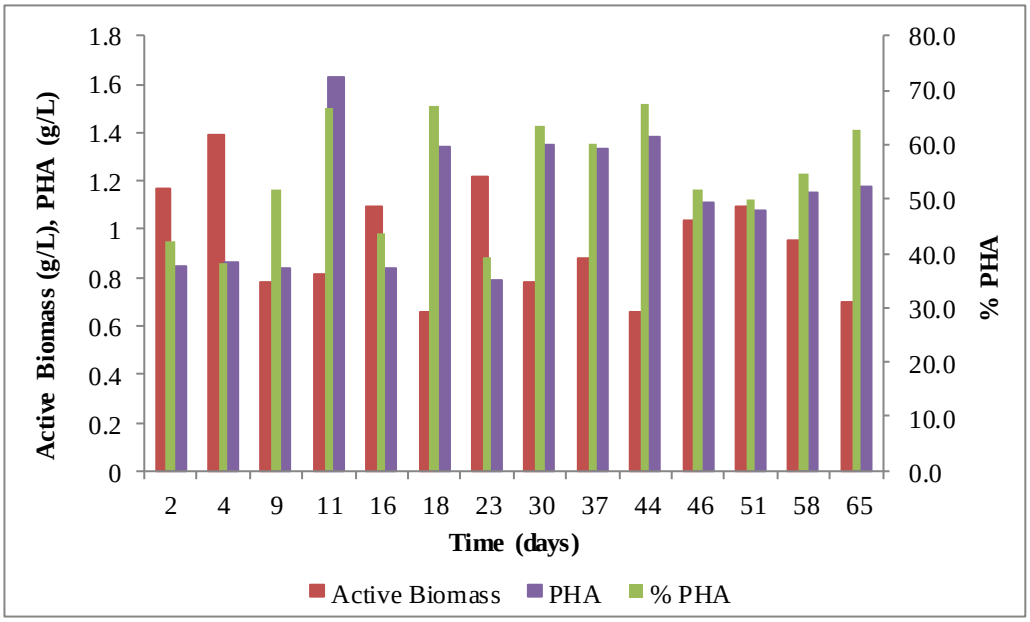


Figure 16: PHA and Active Biomass concentration and % PHA obtained during the SBR operational period

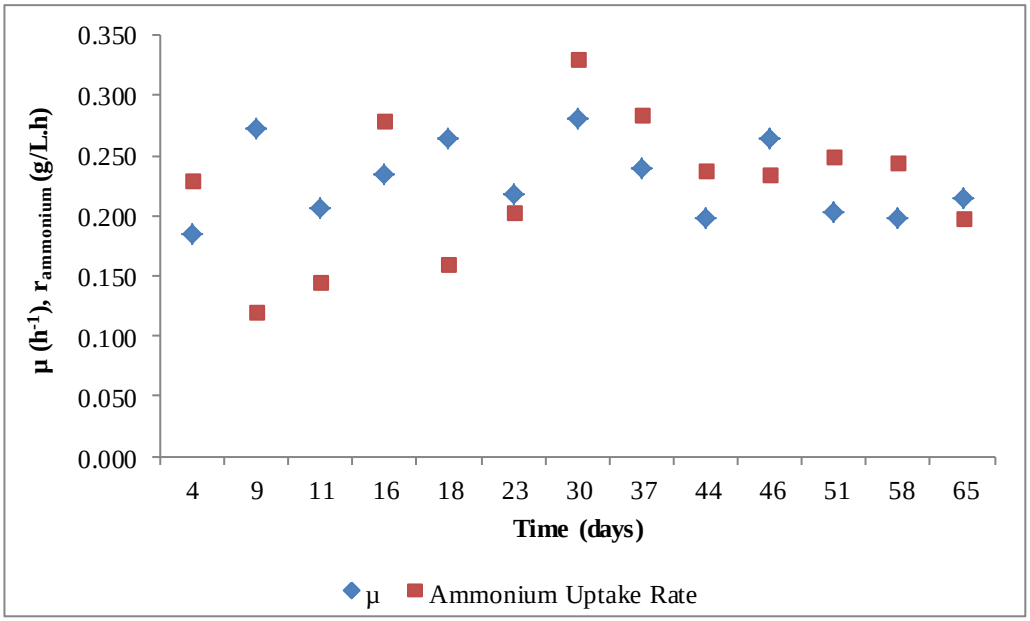


Figure 17: μ and Ammonium uptake rate during the operational period of the SBR.

Evaluating the entire reaction operation, it is possible to establish a relation between biomass growth and PHA production, since the two processes usually compete for the carbon sources (Serafim *et al.* 2008). On the first days of operation, considerable fluctuations on the amount of PHA produced can be observed. On this period also biomass growth showed some instability confirmed by the specific growth rate observed on Figure 17. By the fifth week, 30th day, a more stable polymer production was verified. During the reactor operation, the amount of total biomass (SS) varied in the range of 2.01 ± 0.22 g/L, alongside with the variation of PHA that corresponded to an average value of 1.12 ± 0.26 g/L. The ammonium uptake rate was also determined during the operational period of the SBR (Figure 17). Its highest value was recorded on the 30th day of operation, 0.0060 g/L.h, and it was possible to see stabilization on the values starting on this day which corresponds to the biomass concentration stabilization (Figure 16).

Comparatively to the previous results, the values of specific growth rate, presented in Figure 17, don not show a tendency by the 5th week of operation, when the culture stabilized. The values of the specific growth rate ranged between 0.185 – 0.281 h^{-1}, and the highest value was obtained in the 30th day, 0.281 h^{-1}.

Figure 16 also shows the percentage of PHA cell content during the entire operational period. The values varied within the range of 54.2 ± 10.6 %, with a maximum fixed in 67.6 % (44th day) and a minimum at 4th day corresponding to 38.2 %. Within the

zone of a more stable production (between the 30th and 65th days), the percentage of PHA varied in the range of 57.5 ± 5.9 %, and in this period of time, the maximum of PHA accumulation was registered. Unfortunately, the analytical method used in the present work did not allow for the identification of PHA monomers. This identification is usually done by gas chromatography, GC, after methanolysis of the obtained monomers (Serafim *et al.* 2002). The applied method only accounts for the total amount of PHA and presents problems of accuracy on samples with low concentration (data not shown). Moreover some cellular compounds can interfere with spectrophotometric measurements at 235 nm, although variation of these compounds is not expected (Karr *et al.* 1983). Karr *et al.* (1983) applied this method to analyze PHB production by *Rhizobium japonicum* from soybean and measured the results using HPLC and by spectrophotometry. The obtained results were compared with those obtained by GC. The methods that allowed for fractionation, HPLC and GC, gave results with a difference of 4.5 % between them. While methods which do not, as spectrophotometric, led to values 5.6 % higher than the GC results (Karr *et al.* 1983). For these reasons the PHA values obtained in this work can be considered slightly higher than the real values.

Nevertheless the obtained results showed a clear selection of a MMC with a good PHA storage capacity, even with problems with method accuracy and the observed fluctuations. These fluctuations could be a result of the different bacterial groups' composition. The previous evidences could be explained by the fact that within the MMC, the bacterial groups can vary in their relative abundance along the operational period as observed by Lemos *et al.* (2008). Moreover, the results obtained in this work are in the same range of best results obtained using real complex waste as reviewed by Serafim *et al.* (2008). It should be noted again, that the highest PHA content matched with the low biomass concentration, and specific growth rate, as stated in the literature (Serafim *et al.* 2004). This was expected because PHA production competes directly with the bacterial growth for the substrate, leading to low biomass concentration with high PHA accumulation. However, this relation was not observed as a tendency in the present work. This might be due to the complexity of the raw material used as substrate and the different bacterial groups consume the different carbon sources present without competition between them for the substrate.

4.1.2. SBR CYCLE

As described in the previous chapter, an SBR was operated during 10 weeks with the objective of selecting a culture able to produce and accumulate PHA using HSSL as carbon source. During this period, several parameters were monitored along the SBR cycle, namely, acetic acid, xylose and ammonium uptake, PHA production, oxygen consumption, pH and temperature evolution. Figure 18 shows the typical values obtained along a SBR cycle, on the 37th day of operation.

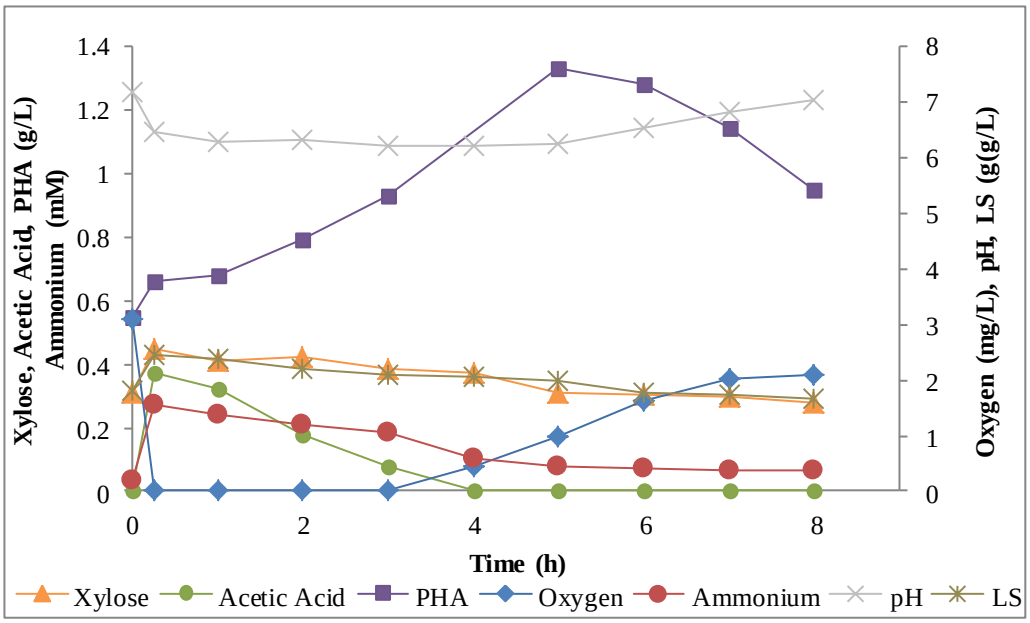


Figure 18: SBR cycle at the 37th (6th week) day of operations.

Along the cycle, pH presented two distinct behaviors: after the feeding (t = 15min), it decreased until 6.19 at 4 h, followed an increase over the cycle reaching the initial valor, 7.15. Although the temperature tendency was the same in every cycle, during the entire operation the temperature dropped 4 ºC. However, this decrease resulted from the room temperature decrease along the operation and did not seem to have influenced the growth rate or the consumption rate of substrates by the culture. This could be an advantage of this system since at higher scale the temperature control of this type of processes is a major contribution to production costs.

The concentrations of acetic acid, xylose, lignosulphonates (LS) and ammonium decreased along the cycle. Acetic acid was totally depleted at 4.0 h while ammonium was present in the whole cycle. Acetic acid was consumed at 0.110 g/L.h. The ammonium

uptake rate was faster before acetic acid depletion, 0.177 g/L.h, than after, 0.074 g/L.h. Xylose was slightly consumed while acetic acid was being consumed and it stopped 1 h after acetic acid depletion, always at the same rate. LS were consumed along the cycle always at the same rate, 0.098 g/L.h. Similar profiles for carbon sources were observed by Moita and Lemos (2011) when using pyrolysis products to select a PHA-storing MMC (Moita and Lemos 2011).

Since the different carbon sources present were consumed in different ways, the definition of the feast and famine phase could not be based on the carbon sources evolution. The time of the exhaustion of the carbon source is usually characterized by the increase of dissolved oxygen in the medium, but in this case it only reacted to the acetic acid. This could be a consequence that acetic acid can be considered as a readily biodegradable substrate and is consumed at a higher rate than xylose and LS, consuming more oxygen (Dircks *et al.* 1999).

In average, considering only acetic acid and oxygen consumption, during the reactor operational period, the feast phase had the duration between 3 to 4 hours, and the famine phase between 7 to 8 hours. Nevertheless, this feast/famine phase it is applicable to organisms that consume acetic acid, not being, however, a true feast/famine phase for the organisms that consume other carbon sources present in HSSL, like xylose and LS.

Regarding to the PHA accumulation, as a primary product it is synthesized during the cell growth. Supposedly, the maximum of PHA should be reached when the VFAs were fully consumed (at the end of the feast phase), yet, the maximum of the PHA was only achieved one hour after the exhaustion of the acetic acid, 1.33 g/L. This suggests that other carbon sources were also involved in the PHA accumulation. After this point, the amount of PHA decreased due to its consumption by bacteria for their maintenance and growth (the ammonium keeps being consumed, but at a low rate), although xylose and LS were still present and being consumed. This behavior was also observed by Moita and Lemos (2011) using pyrolysis by-products as carbon substrate.

Despite consumption of xylose, at end of the cycle a significant amount remained in the reactor, accounting for 62 % of the initial value. It means that the effluent of the SBR could serve as substrate for other biological processes that use this pentose as carbon source. Moreover the acetic acid and LS consumption would help for a subsequent utilization of the effluent since are known as inhibitors of many biological processes,

namely bioethanol production from xylose by *Scheffersonmyces stipitis* (Pereira *et al.* 2012). The use of HSSL for bioethanol production implies a preliminary detoxification step which can be expensive, if ionic-exchange resins are used (Xavier *et al.* 2010) or slow if fungal biomass is used (Pereira *et al.* 2012). The utilization of a PHA-storing MMC could decrease the price and quicken this detoxification step, since a valuable production is obtained in 12 hours. The obtained results showed that PHA production could be integrated with bioethanol fermentation by yeast in a HSSL-based biorefinery.

4.2. KINETIC TESTS

In order to understand in more detail the conversion of some components of HSSL by the selected MMC to PHA or growth and the role of the different carbon sources present in HSSL, kinetic batch tests were performed. The selected MMC was collected from the SBR and inoculated to a batch reactor connected to a respirometric system that allowed for the determination of oxygen consumption. This kinetic study also included a test with the objective of maximize PHA production by submitting the MMC to consecutive feeding pulses of HSSL. These tests also encompassed the study of effect of the two major carbon sources present in the HSSL, acetic acid and xylose, separated and mixed, in mineral medium. The presence of the ammonium was also evaluated since its limitation usually triggers PHA production by MMC mixed cultures. For all tests several kinetic and stoichiometric parameters were calculated, namely specific acetic acid (q_{acetic}) and xylose (q_{xylose}) uptake, growth (μ) and PHA production (q_{PHA}) rates, and storage ($Y_{(PHA/S)}$), growth ($Y_{(X/S)}$) and respiration ($Y_{(O2/S)}$) yields on substrate (acetic acid and xylose) and respiration yield on biomass ($Y_{(O2/X)}$).

4.2.1. ASSAY WITH HSSL

The first test performed was the study of the kinetics of PHA production from HSSL components and the results obtained are shown in Figure 19 and the kinetic and stoichiometric parameters are presented in Table 9. In this assay the biomass was diluted 1 time in order to keep oxygen values above 10 % of saturation and allow for the calculation of oxygen uptake rates (OUR). The amount of HSSL was the same as in the SBR. As observed in the SBR, the acetic acid was totally consumed after two hours. Unlike of what was usually observed in SBR, during the test the xylose was totally depleted after 4.5 h but at a slower rate (0.072 g/L.h) than the acetic acid (0.282 g/L.h). As in SBR, oxygen reacts differently to these substrates. The oxygen dissolved clearly increased and the OUR decreased when the acetic acid was exhausted while the differences were smoother when xylose was depleted. The differences observed on carbon source consumption probably resulted from the amount of oxygen available, since during the SBR the amount of oxygen often dropped to zero resulting in limiting conditions, while during the kinetic test, the minimum value measured was 2.6 mg/L.

PHA production occurred mainly from acetic acid and only a small amount from xylose. PHA reached its maximum, 1.08 g/L that corresponded to 63.3 %, at 2.9 h. After this period PHA consumption started, probably, for growth and cellular maintenance, even with xylose being consumed. Considering the total biomass concentration it increased until the steady state that has its beginning at the end of the xylose consumption. After a while it started to decrease mostly due to the PHA consumption for the cellular maintenance.

This study is not totally completed since the monomeric composition of the PHA produced was not determined and the evolution of COD in external medium was not measured due to technical limitations as stated in previous sections. In order to determine the amount of total carbon present in HSSL that could contribute besides acetic acid for PHA production.

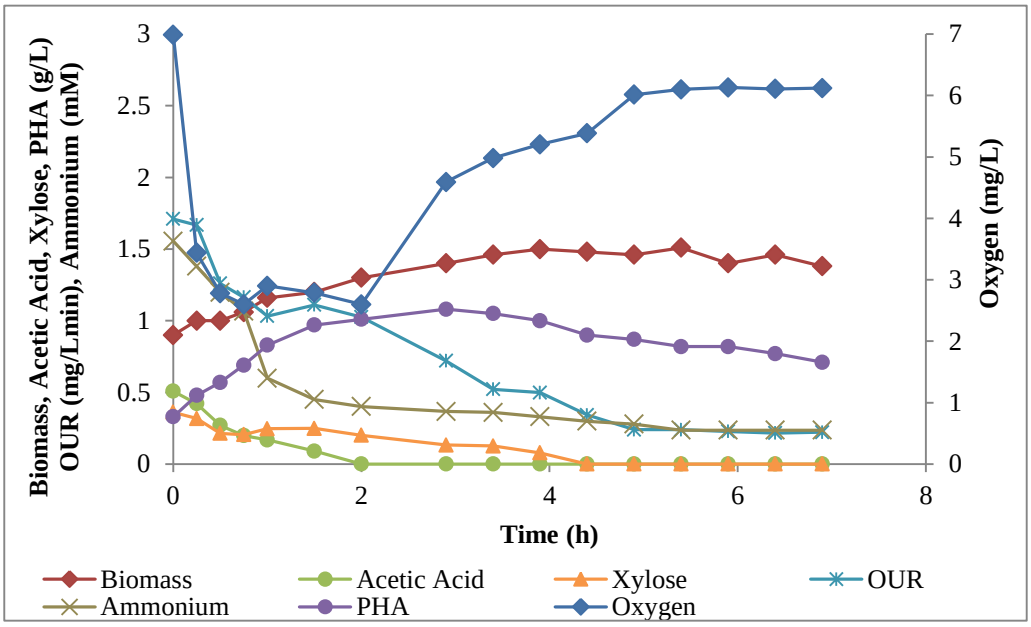


Figure 19: Evolution of Oxygen, Biomass, PHA, Ammonium, Acetic Acid and Xylose concentration in kinetic test using HSSL as substrate. Test performed at the 2nd week of operation.

Table 9: Kinetics parameters obtained in the different tests performed.

Test	X_{active} (g/L)	Acetic $Acid_i$ (g/L)	$Xylose_i$ (g/L)	$Y_{(O2/X)}$ (gO_2/gX)	$Y_{(O2/S)}$ (gO_2/gS)	$Y_{(X/S)}$ (gX/gS)	$Y_{(PHA/S)}$ (gPHA/gS)	μ (h^{-1})	$-q_{Ac}$ (gAc/gX.h)	$-q_{xyl}$ (gXyl/gX.h)	PHA_i / PHA_f (%)
HSSL	0.9	0.361	0.508	0.955	0.220	0.230	0.817	0.143	0.401	0.186	36.7 / 63.3
HSSL (- NH_4)	0.77	0.718	0.512	---	0.160	---	0.492	---	0.584	0.058	32.0 / 58,4
Acetate	0.32	0.630	---	0.571	0.340	0.270	0.850	0.09	1.122	---	25.0 / 63.4
Xylose	1.84	---	1.359	0.704	0.211	0.299	0.572	0.119	---	0.185	9.8 / 31.5
Acetate + Xylose	1.48	1.180	0.783	0.465	0.626	0.202	0.829	0.200	0.498	0.084	15.9 / 62.0
HSSL (5pulses)	1.05^a	0.209^a	0.517^a	---	0.282^a	---	0.994^a	---	0.301^a	0.080^a	$6.3 / 19.5^a$
	1.18^b	0.174^b	0.188^b		0.483^b		0.880^b		0.223^b	0.031^b	$3.9 / 12.7^b$
	1.23^c	0.266^c	0.221^c		0.353^c		0.640^c		0.328^c	0.070^c	$8.2 / 21.0^c$
	1.29^d	0.205^d	0.218^d		0.457^d		0.968^d		0.241^d	0.039^d	$9.2 / 21.5^d$
	1.49^e	0.226^e	0.312^e		0.458^e		0.610^e		0.300^e	0.034^e	$9.1 / 27.0^e$

a – first pulse, b - second pulse, c - third pulse, d - fourth pulse, e - fifth pulse, i - initial valor, f - final valor

The yields determined based on xylose and acetic acid consumption clearly show that other carbon sources are involved in PHA production (0.817 gPHA/gS), growth (0.230 gX/gS) and oxygen consumption (0.220 gO_2/gS), since the sum of these values are clearly above 1.

4.2.2. STUDY OF EFFECT OF AMMONIUM

One of the main parameters measured in this process is the ammonium present in the external medium that usually acts as the trigger of PHA production by MMC (Serafim *et al*. 2004). In this test the MMC was inoculated with mineral medium with HSSL but lacking a source of ammonium. The results obtained are shown in Figure 20 and the kinetic and stoichiometric parameters in Table 9.

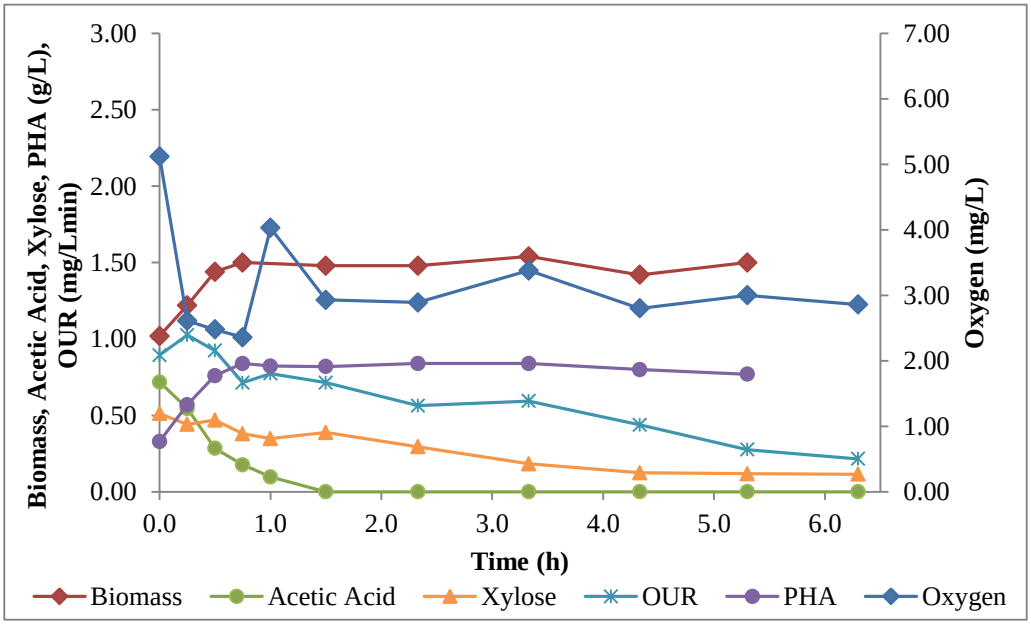


Figure 20: Evolution of Oxygen, Biomass, PHA, Acetic Acid and Xylose concentration in kinetic test using HSSL without ammonium as substrate. Test performed at the 8th week of operation.

In this test, the acetic acid was fully consumed but the xylose only suffered a small decrease. Also the specific uptake rates were different than those observed in the previous test, $-q_{acetic}$ raised to 0.584 gAc/gX.h and $-q_{xylose}$ decreased to 0.058 gXyl/gX.h. This could be explained by the possible faith of the two carbon sources. The decrease of the oxygen dissolved in the medium and the increase in OUR values were lower than observed when ammonium was supplied. Without the ammonium, which is essentially for cell growth and

replication, it can be assumed that most of the carbon consumed was redirected only to PHA production and cell maintenance. The biomass curve followed PHA evolution which is evidence that no active biomass was formed. This test also confirmed that HSSL is a substrate poor in nitrogen.

It could be assumed that acetic acid is used by microorganisms that can grow and store PHA and xylose is mainly utilized by microorganisms that grow and only a few are able to accumulate PHA. Comparing the amount of polymer, the maximum content obtained was 58.4 % of cell dry weight, more 26.4 % than at the beginning of the experiment. This value was similar to the obtained in the experiment with ammonium (ΔPHA = 26.6 %). This is in accordance to what was observed by Serafim *et al.* (2004) that stated the absence of ammonium did not enhance significantly PHA production by MMC under ADF conditions but a small value of ammonia present in the medium.

4.2.3. STUDY OF THE EFFECT OF ACETIC ACID AND XYLOSE

In order to understand the role of xylose and acetic acid in the metabolism of the selected MMC, three more kinetic tests with synthetic medium containing acetic acid, xylose and a mixture of both were performed. In the assay performed with acetic acid as sole carbon source (Figure 21), the depletion of the carbon source was confirmed by the increase of the dissolved oxygen and decrease of OUR.

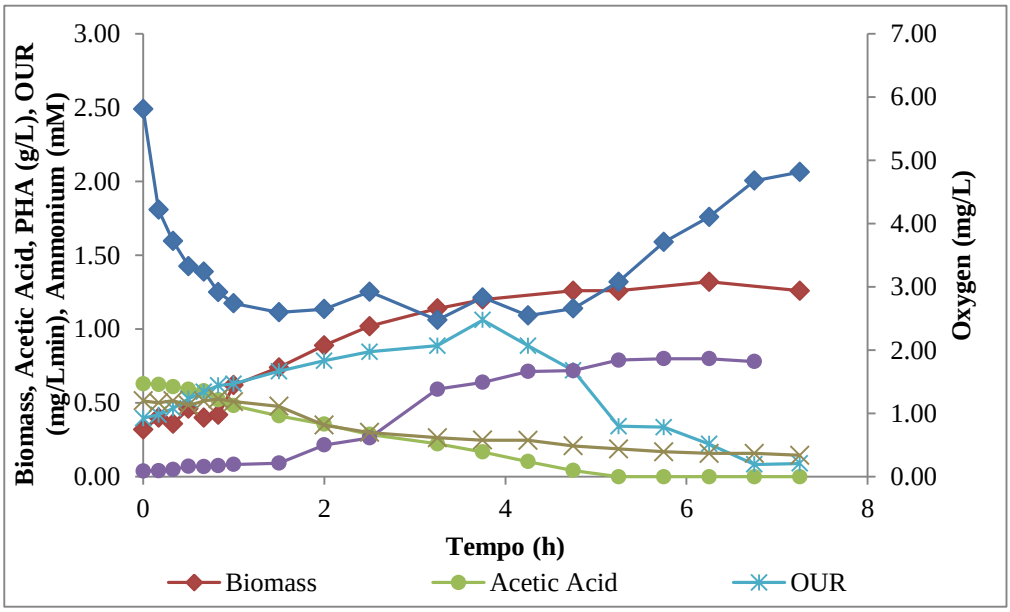


Figure 21: Evolution of Oxygen, Biomass, PHA, Ammonium and Acetic Acid concentration in kinetic test using Acetic Acid as sole carbon source. Test performed at the 3rd week of operation.

In this test, a lag phase of 1.5 h in PHA production was observed probably due to the adaptation of the MMC, coming from medium containing HSSL, to the mineral medium.

In the assay performed with xylose as sole carbon source (Figure 22), there was a problem in preparing the medium and the amount of xylose added was higher than expected. The xylose was present through the all experiment and it was never exhausted. Nevertheless, as in the experiment with acetic acid, a lag phase of 1 h in PHA production was observed.

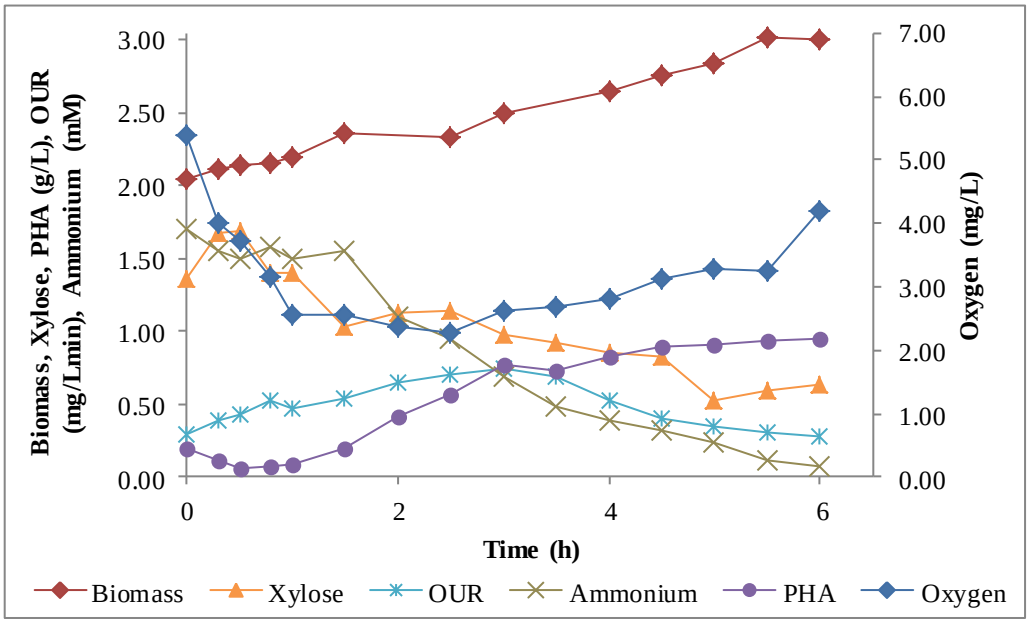


Figure 22: Evolution of Oxygen, Biomass, PHA, Ammonium and Xylose concentration in kinetic test using only Xylose as sole carbon source. Test performed at the 4th week of operation.

In experiment with acetic acid the lowest specific growth rate, 0.090 h^{-1} and the maximum of PHA accumulation, 63.4 %, that corresponded to one of the highest values of variation of Δ%PHA of 38.6 % were obtained (Table 9). While in experiment with xylose, the specific growth rate was higher, 0.119 h^{-1}, and the lowest amount of PHA was obtained, Δ%PHA of 21.3 %. Regarding to the yields, $Y_{(PHA/S)}$ of acetic acid assays was also the highest obtained in the present work, 0.850 gPHA/gS while the highest $Y_{(X/S)}$, 0.299 gX/gS was obtained in the assay with xylose. In both assays the amount of oxygen consumed per biomass was lower than in the assay with HSSL, probably due to the absence of toxic compounds in the medium and other compounds that were easily

oxidized. These results confirm the possibility of PHA production by the MMC from acetic acid and xylose. Moreover it is possible to assess that fraction of xylose-consuming organisms that produces PHA is lower than in acetic acid consuming organisms.

Finally in the test with a mixture of acetic acid and xylose (Figure 23), the lag phase observed in PHA production was shorter than in the two other assays. The pattern recorded was similar to the observed during the cycles with HSSL: acetic acid was depleted (0.498 gAc/gX.h) while xylose was slowly consumed (0.084 gXyl/gX.h).

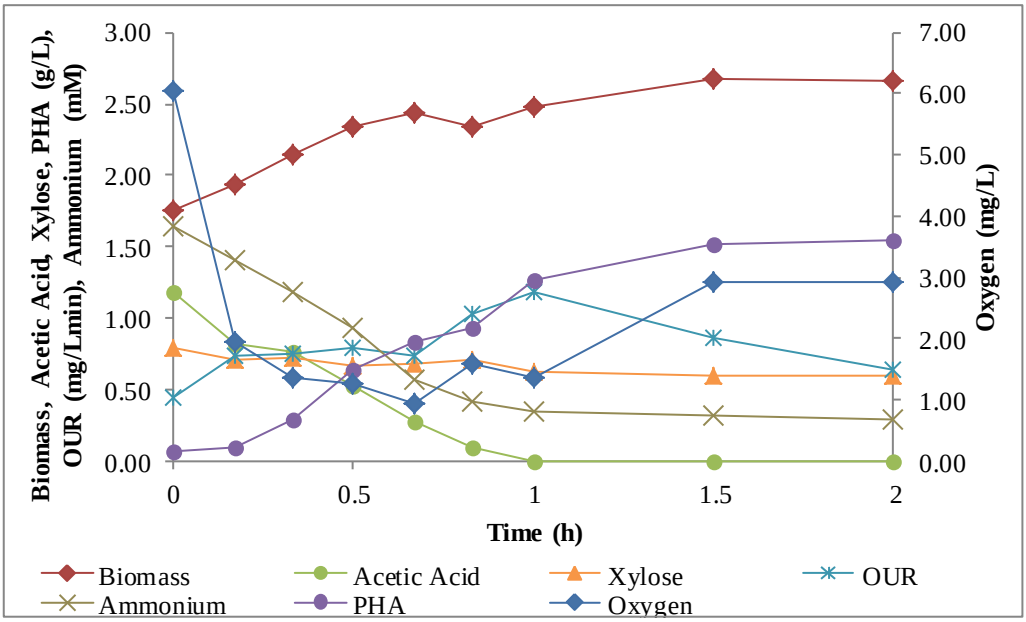


Figure 23: Evolution of Oxygen, Biomass, PHA, Ammonium, Acetic Acid and Xylose concentration in kinetic test using a mixture of acetic acid and xylose as carbon source. Test performed at the 5th week of operation.

The amount of PHA obtained was one of the highest obtained, Δ%PHA of 46.1 %. At the end of this test, PHA was extracted but the composition was not possible to determine and its characteristics were not assessed due to technical impossibilities.

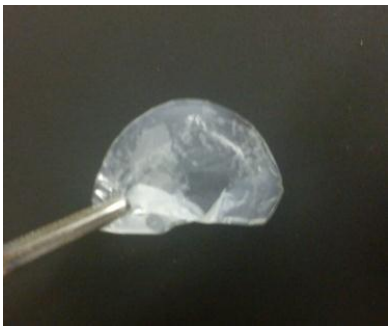


Figure 24: PHA extracted from the culture at the end of test with a mixture of acetic acid and xylose as carbon

4.2.4. ACCUMULATION ASSAY WITH HSSL

Finally an accumulation assay with five pulses of HSSL mixed with mineral medium was performed (Figure 25). However the strategy chosen was not successful since the time between the depletion of acetic acid of the previous pulse and the following one was too long allowing for excessive consumption of PHA. Moreover all the pulses contained ammonia and the non-limiting oxygen conditions allowed cells to growth using the stored PHA. For these reasons the maximum amount of PHA storage content obtained was 27.0% in the last pulse corresponding to a production of Δ%PHA of 17.9%. Future accumulation tests should be performed with limiting conditions of ammonium and oxygen.

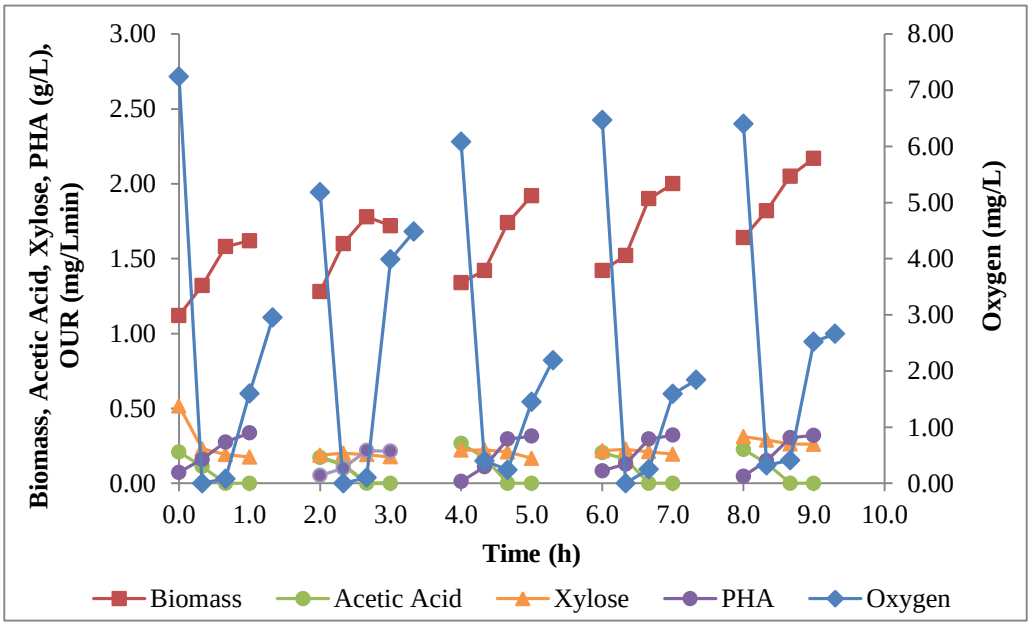


Figure 25: Evolution of Oxygen, Biomass, PHA, Acetic Acid and Xylose concentration in kinetic test during the five pulses of feeding. Test performed at the 10th week of operation.

4.3. MICROBIAL COMMUNITY CHARACTERIZATION

4.3.1. MORPHOLOGICAL ANALYSIS

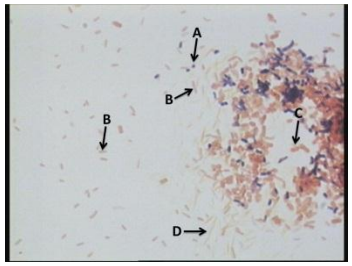
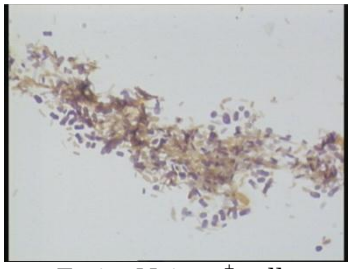
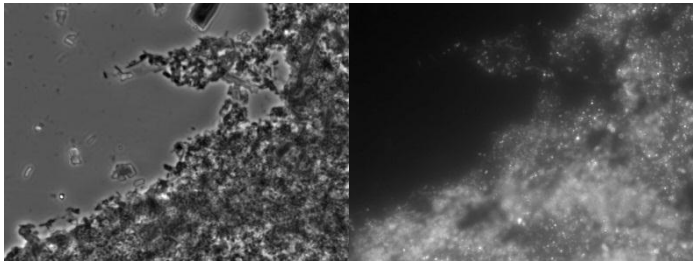
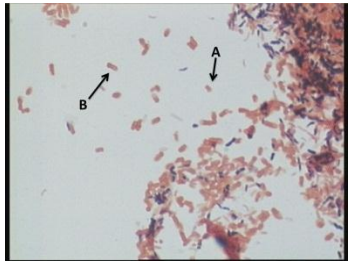
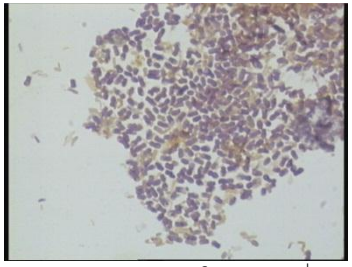
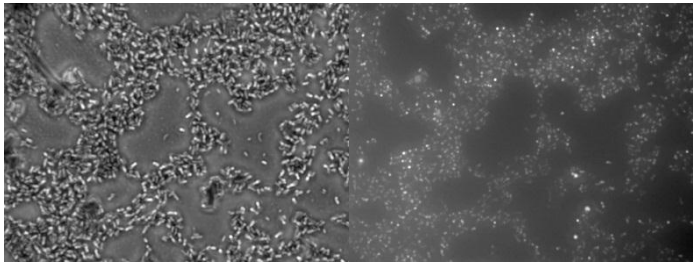
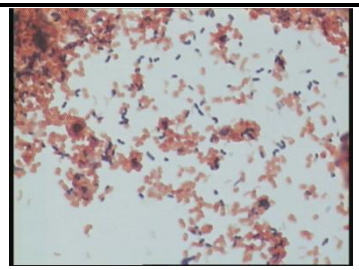
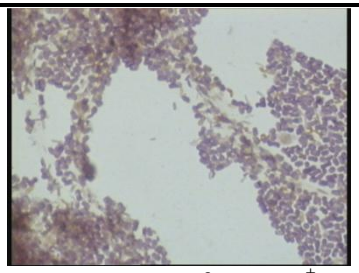
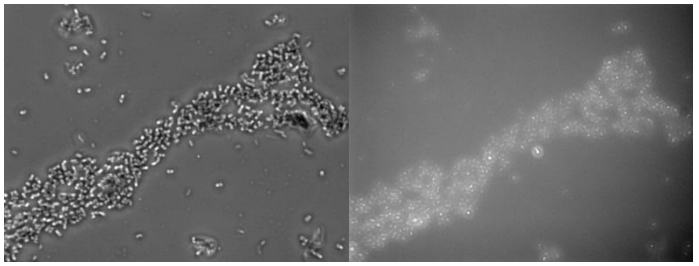
As previouly mentioned, with the purpose of optimizing the reactor operation and the efficiency of the process, the study of the microbial community is required. In this project, the bacterial community selected in the SBR was morphologically and taxonomically characterized along the operational time. The main morphotypes present in the selected MMC were preliminary described by phase contrast microscopic analysis and using biological stain procedures such as Gram, Neisser and Nile blue, a specific staining for PHA inclusions. In all observations made it was confirmed the strong tendency of bacteria to form aggregates easily visualized in the images taken.

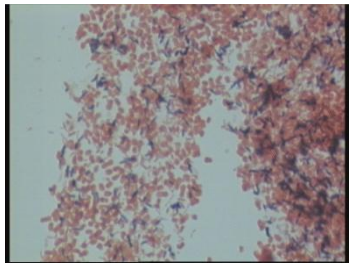
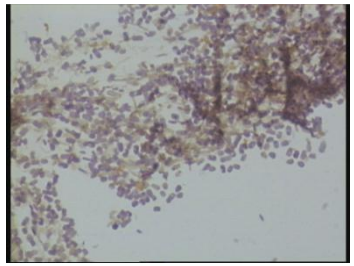
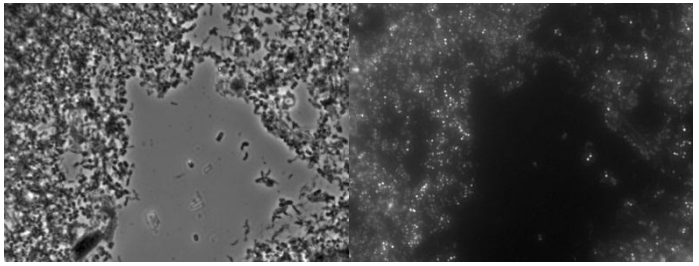
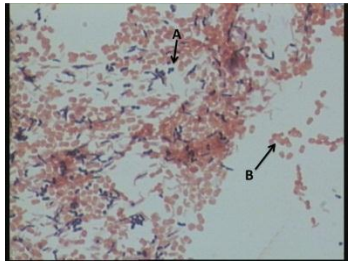
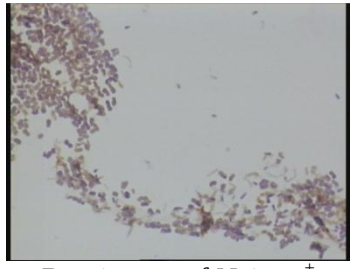
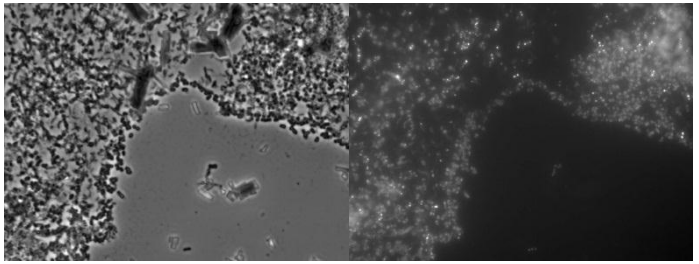
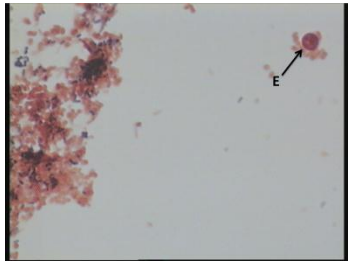
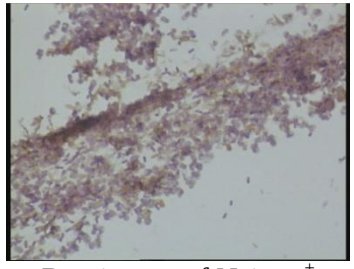
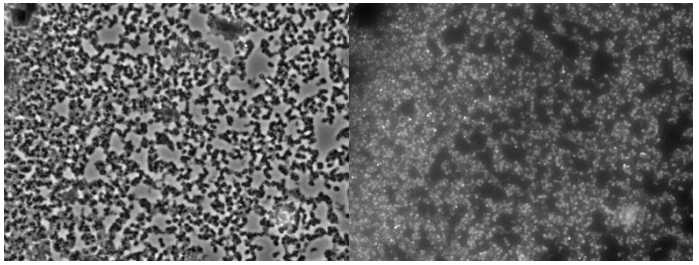
In Table 10 the results obtained from Gram, Neisser and Nile blue staining are summarized. Since the beginning of operation under ADF condition, it was easily perceptible the presence of inclusions bodies of PHA by the presence of brilliants dots in the cells after Nile Blue staining procedure under epiflurescence. Even though the presence of PHA granules was detected, it was not possible to define the relative abundance since ethanol was used to fix the cells and it usually dissolves the inclusion bodies.

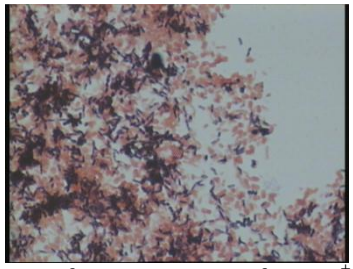
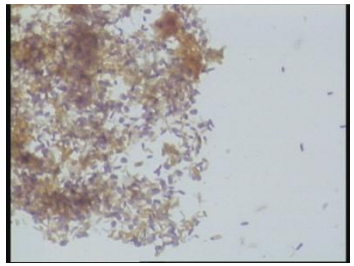
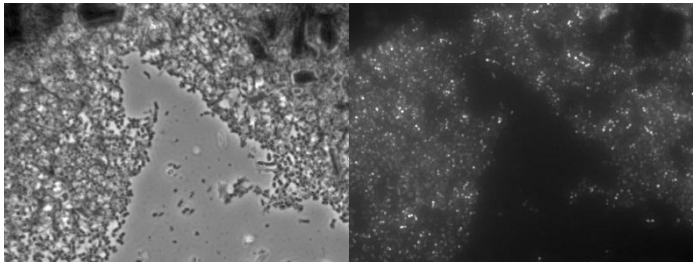
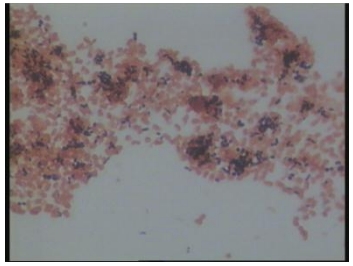
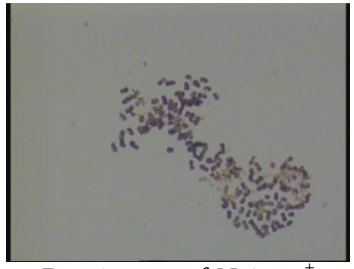
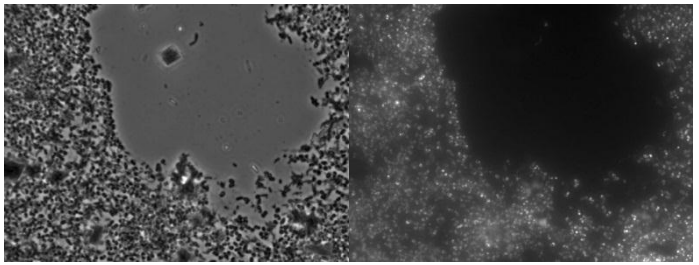
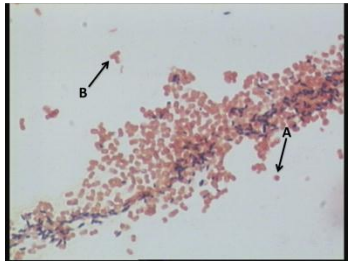
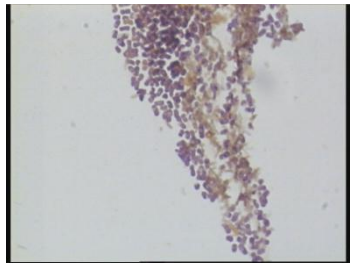
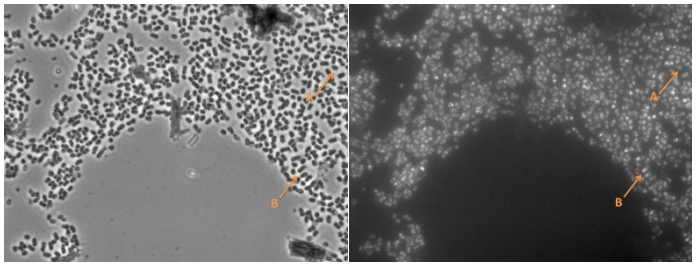
Resorting to Gram staining, the dominant morphotype during the entire operating period was the Gram negative ($Gram^-$) cells. With this staining, it was also possible to follow the evolution of cells morphology. The dominant morfologies were *cocci* and *bacilli* with the presence of few *coccobacilli*. Along with the observations of Nile Blue staining and phase contrast, it is possible to state that *cocci* and *bacilli* were the main PHA accumulating bacteria in the culture.

Neisser staining allowed also to follow the evolution of the culture. At the end of the operating period, the Neisser positive cells ($Neisser^+$) and Neisser negative cells ($Neisser^-$) were, aproximately, in the same proportion. It should be noted that $Neisser^+$ cells were totally stained without the tipically dark spots at the edge of the cells, typical of phosphate accumulating organims. Moreover with Neisser staining, in some cases, the PHA inclusion bodies in some cells were perfectly seen.

Table 10: Pictures of Gram, Neisser and Nile Blue staining of the samples retrieved during reactor operations.

Sample	Morphotypes	Gram Stain	Neisser Stain	**Nile Blue Stain** (Phase contrast/Epifluorescence)
Sample A (2nd week)	**3 main morphotype:** *Cocci* (A); (di)*bacilli* (B); *coccobacillus* (C) Significant amounts of long, thin and curve *bacilli*; visualization of *streptobacilli* (D)	 Dominance of Gram^{-}	 Entire Neisser^{+} cells	
Sample B (3rd week)	Disappearance of *streptobacilli*; Decreasing of long, thin and curve *bacilli*; Establishment of 2 main morphotypes: *cocci* and *bacilli*	 Dominance of Gram^{-}	 Dominance of Neisser^{+}	
Sample C (4th week)	**2 main morphotypes**: *cocci* and *bacilli* Trace amounts of long, thin and curve *bacilli*; (*diplobacilli* – Gram^{+})	 Dominance of Gram^{-}	 Dominance of Neisser^{+}	

Sample D (5th week)	**2 main morphotypes**: *cocci* and *bacilli* Disappearance of long *bacilli*.	 Dominance of Gram^{-}	 Increase of Neisser^{-}	
Sample E (6th week)	**2 main morphotypes**: *cocci* and *bacilli*	 Dominance of Gram^{-}	 Dominance of Neisser^{+}	
Sample F (7th week)	**2 main morphotypes**: *cocci* and *bacilli* (Visualizations of big, round and well defined cells (E))	 Dominance of Gram^{-}	 Dominance of Neisser^{+}	

Sample G (8^{th} week)	**2 main morphotypes**: *cocci* and *bacilli* (Disappearance of the cells previously recorded (E))	 Significant increase of Gram^{+}	 New increase of Neisser^{-}	
Sample H (9^{th} week)	**2 main morphotypes**: *cocci* and *bacilli*	 Dominance of Gram^{-}	 Dominance of Neisser^{+}	
Sample I (10^{th} week)	**2 main morphotypes**: *cocci* and *bacilli* (significant amount of *coccobacillus*)	 Dominance of Gram^{-}	 Considerable amount of Neisser^{-}	 Clearly visualization of PHA granules in phase contrast, further highlighted by the epifluorescence microscop

4.3.2. FISH ANALYSIS

The previous evaluation was followed by a taxonomic analysis by FISH. An initial approach, without a pretreatment before probe applying it was only possible to identified 40 % of bacteria out of the total cells. The pretreatment, described in the previous section, conjugated with the application of beads to reduce the degree of aggregation, enhanced the probe penetration in the cells in 40 - 45 %.

In a first step, specific probes for the main phyla within bacteria domain were applied. This step revealed that the organisms were mostly bacteria and no *Archaea* was found in the culture. Microscope observation, under phase constrast, also did not reveal organisms belonging to *Funghi* kingdmon. It is also noteworthy the high activity of the biomass present in the reactor. DAPI staining allowed for the calculation of the ratio between bacteria that hybridized with EUBmix probe and the total DAPI stained cells and result was above 80 %. After this first approach, the identification of bacteria, reported as PHA-storing organism in previous works was performed.

4.3.2.1. BACTERIAL GROUP ANALYSIS

FISH analysis was applied to samples collected on the second (B) and 10^{th} (I) week of operation. *Alpha*, *Beta*, *Gamma*, *Deltaproteobacteria* were retrieved together with cells binding probes for *Flavobacteria*, *Bacteroidetes*, *Sphingobacteria* and *Actinobacteria* on sample B as it is shown in Figure 26.

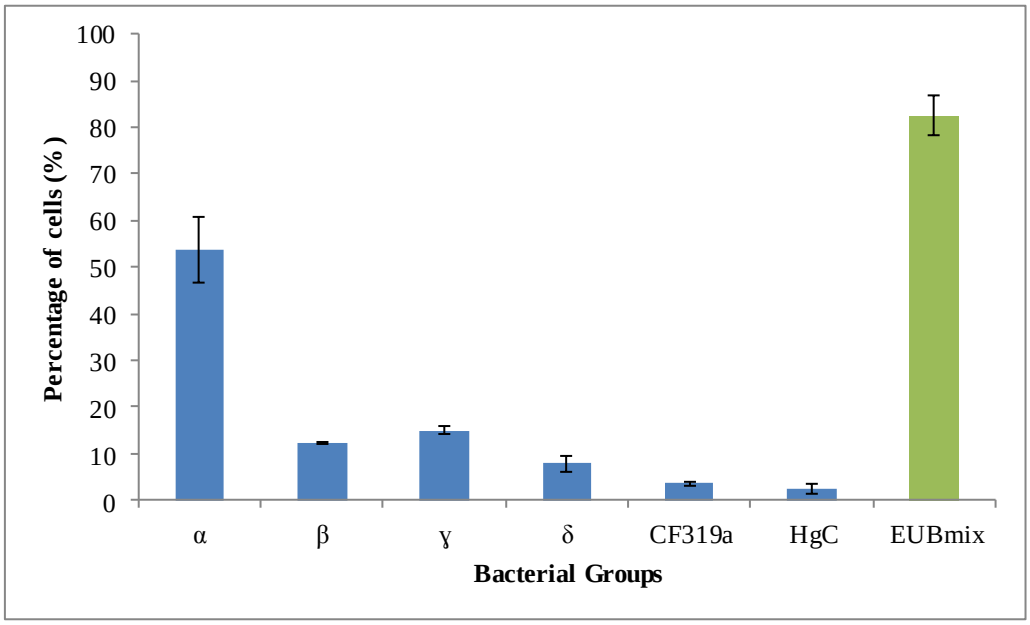


Figure 26: Bacterial Community on sample B (2nd week of operation). α – *Alphaproteobacteria*; β – *Betaproteobacteria*; ɣ - *Gammaproteobacteria*; δ – *Deltaproteobacteria*; CF319a – *Flavobacteria*, *Bacteroides*, *Sphingobacteria*; HgC – *Actinobacteria*; EUBmix – total of biomass identified

FISH analysis confirmed the evolution owing to the operational conditions imposed of the bacterial community previously observed with the different staining techniques. In sample I, the A*lphaproteobacteria* community corresponded to 72.7 ± 4.0 %, an increase of nearly 20 % in relation to sample B (Figure 27). In this sample, the *Sphingobacteria* community was extinguished; the *Deltaproteobacteria* suffered a 5 % decrease to 2.44 ± 0.87 % and the *Betaproteobacteria* (11.1 ± 0.37 %) and the *Gammaproteobacteria* (10.3 ± 0.3 %) did not change significantly.

The evolution of the bacterial community throughout the entire reactor operation is shown in Figure 28. This evolutionary graph shows that by 5th week of operation the community remained stable until the end of the operation, despite minor fluctuations observed. Looking for the reactor performance, Figure 16, can be verified that stabilization of the PHA content and amount of active biomass coincides with the stabilization of the microbial culture, by the fifth week. It is also evident, that in the last week, the small fall in the PHA content also matches the increase in the *Alphaproteobacteria* community.

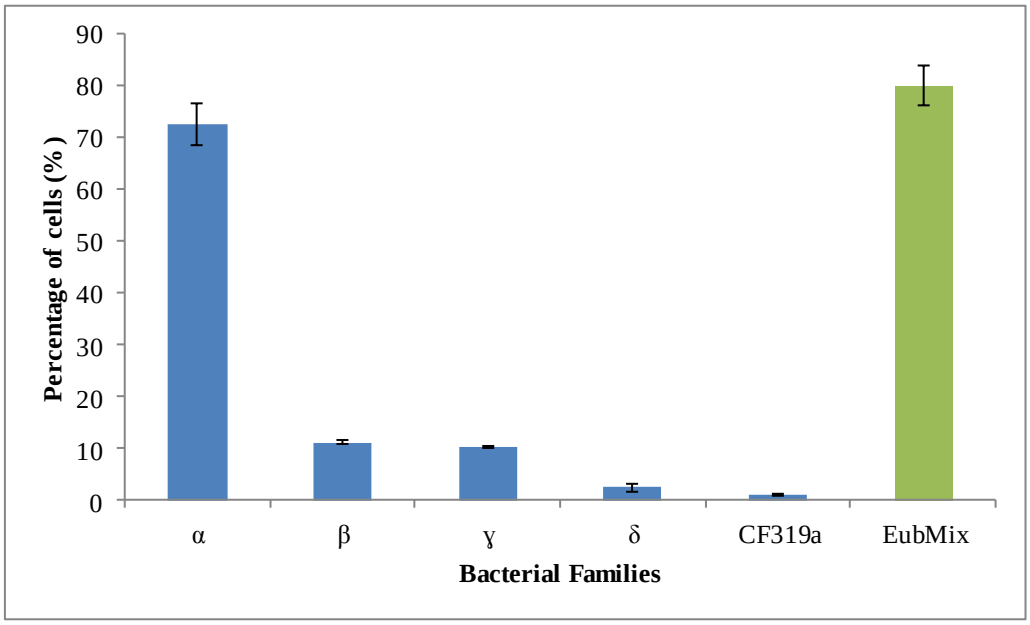


Figure 27: Bacterial Community in sample I (10th week of operation . α – *Alphaproteobacteria*; β – *Betaproteobacteria*; ɣ - *Gammaproteobacteria*; δ – *Deltaproteobacteria*; CF319a – *Flavobacteria, Bacteroides, Sphingobacteria*; EubMix – total of biomass identified

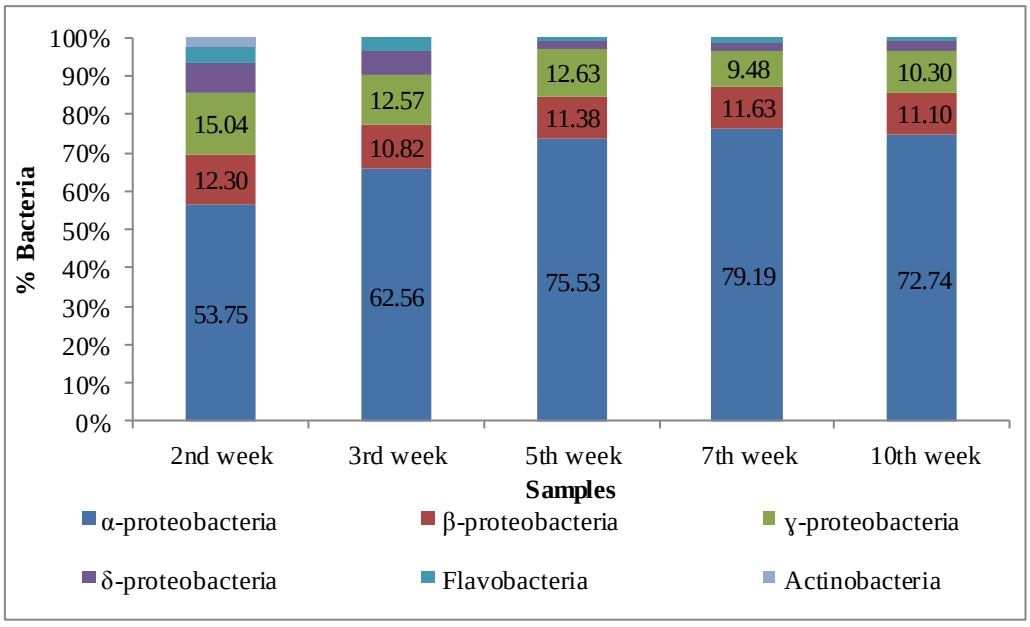


Figure 28: Bacterial community evolution during the reactor operations.

4.3.2.2. ANALYSIS OF FISH PICTURES

Cells belonging to the *Alphaproteobacteria* community (Alf968 probe) are shown as yellow cells in Figure 29, where the blue ones correspond to biomass in which did not occur probe penetration, even after pretreatment. The green cells represent biomass that bound to EUBmix probe for Bacteria Domain. As shown in the picture, the major morphotypes are *cocci* and *bacilli*, which are the main PHA accumulators as shown by the Nile Blue Staining, Table 10.

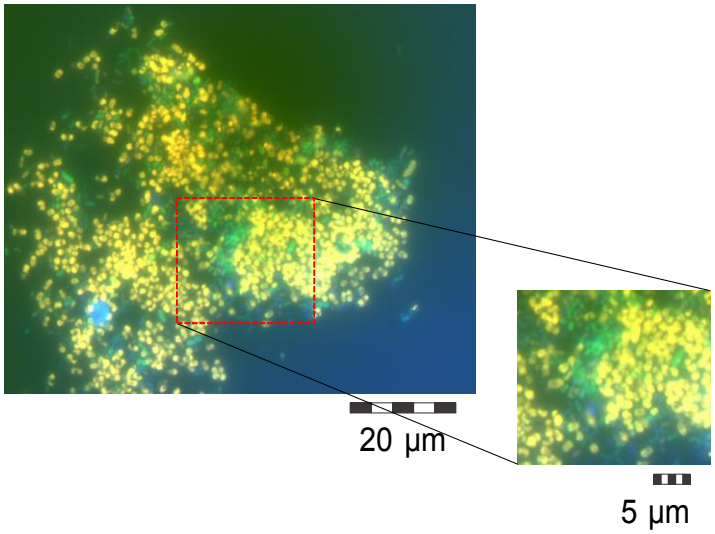


Figure 29: Overlap of FISH pictures and amplification. Yellow cells were hybridized with Alf 968 probe and blue cells represent the remaining biomass that was not hybridized. Probes applied to sample I, last week of operation.

In Figure 30 it is possible to observe another morphotype, *cocobacilli* belonging to *Alphaproteobacteria*,. This morphotype did not seem to be involved in PHA accumulation as shown by the Nile Blue staining.

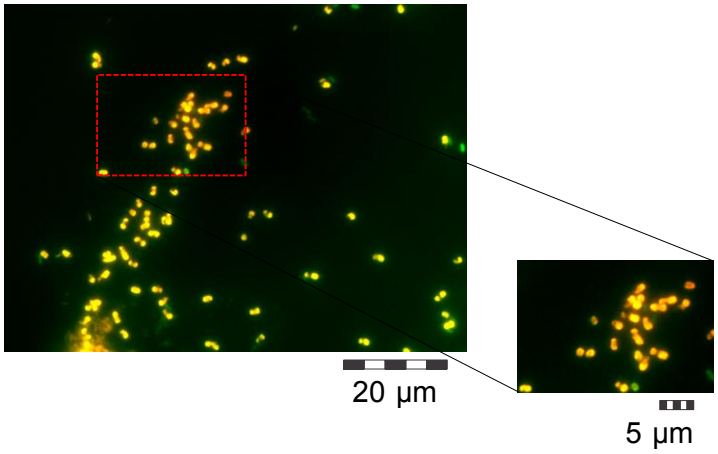


Figure 30: Overlap of FISH pictures and amplification. Green cells were only hybridized by EubMix probe and the yellow cells represent the cells hybridized by EUBmix and Alf968 probes. Probes applied to sample I.

Relatively to the other two main bacterial communities, *Betaproteobacteria* (Figure 31 left) and *Gammaproteobacteria* (Figure 31 right), they were mainly composed by *cocci* and *bacilli*.

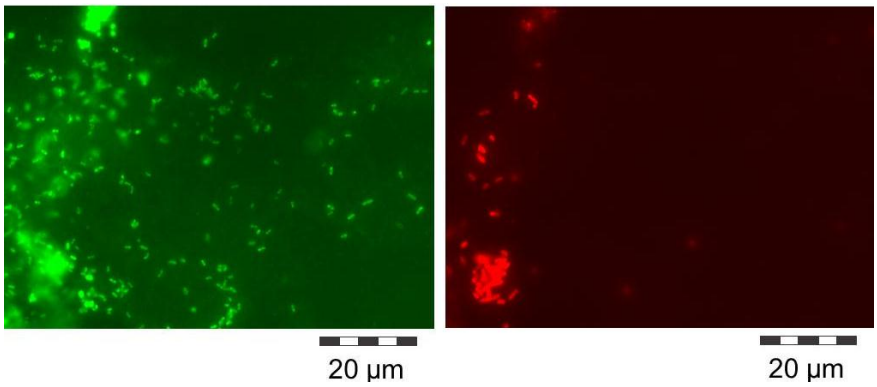


Figure 31: FISH pictures of cells hybridized with Bet42a probe (left picture) and Gam2a (right picture). Probes applied to sample I.

For this analyze, both probes were applied at the same time. Once they differ only in one base, they compete for the binding to 16S RNA. *Beta* and *Gammaproteobacteria* are situated in clusters of microorganisms belonging to *Proteobacteria*. Overlapping these images of Figure 31 with images obtained after Dapi staining, it can be easily gauged that both communities exist in a lesser extent than the *Alphaproteobacteria* community, contrary to what is described in the literature (Dionisi *et al.* 2006; Lemos *et al.* 2008; Moita and Lemos 2011; Jiang *et al.* 2011a). Despite their lowers numbers, both *Beta* and *Gammaproteobacteria* communities are, most likely, responsible for PHA accumulation. In the amplification of Figure 32, it is possible to visualize clear inclusions bodies. These inclusions could be polyphosphate granules or glycogen reserves. However, the Neisser^{+} organisms did not present the tipically dark spots at the edge of the cells (phosphate accumulation) and regarding to glycogen reserves, these are not visible in microscopic observations. Thereby, and like the Nile Blue staining showed that most of the biomass is involved in PHA accumulation, the inclusion bodies vizualized in theses groups could be PHA granules.

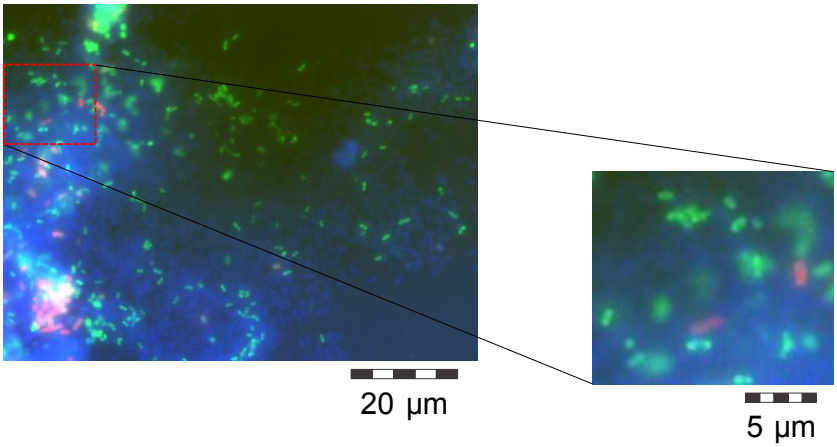


Figure 32: Overlap of FISH pictures and ampliation. Green cells correspond to *Betaproteobacteria*, red to *Gammaproteobacteria* and blue to all remaining biomass not hybridized. Probes applied to sample I.

In the literature some *Deltaproteobacteria* were also described as PHA accumulators (Hai *et al.* 2004). However, in the samples observed, only trace amounts were detected, with a different morphology than that described earlier in Table 10, *cocci* and *bacilli*, Figure 33.

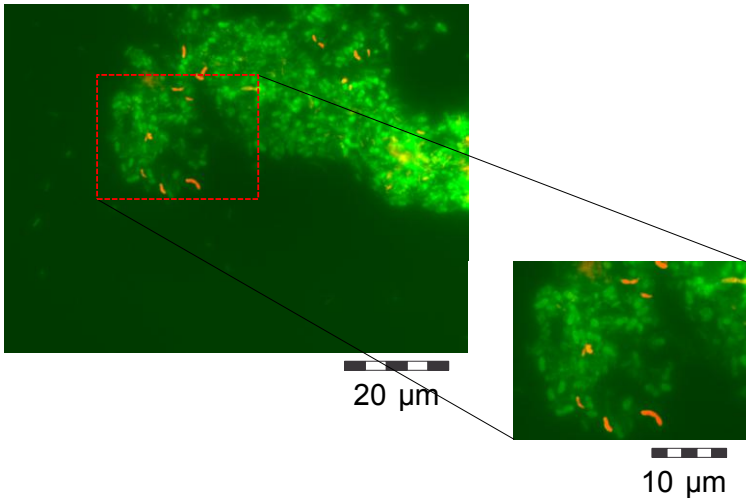


Figure 33: Overlap of FISH pictures and amplification. Green cells were only hybridized by EUBmix probe and the red cells represent the cells hybridized by DeltaMix probe. Probes applied to sample I.

Another probe applied, allowed the identification of *Bacteroidetes* specifically *Flavobacteria*, *Bacteroides*, *Sphingobacteria*. This probe was applied because these bacteria are well described as environmental bacteria, and therefore very susceptible to being present in activated sludge, Figure 34. Similar to *Deltaproteobacteria*, they are present only in trace amounts.

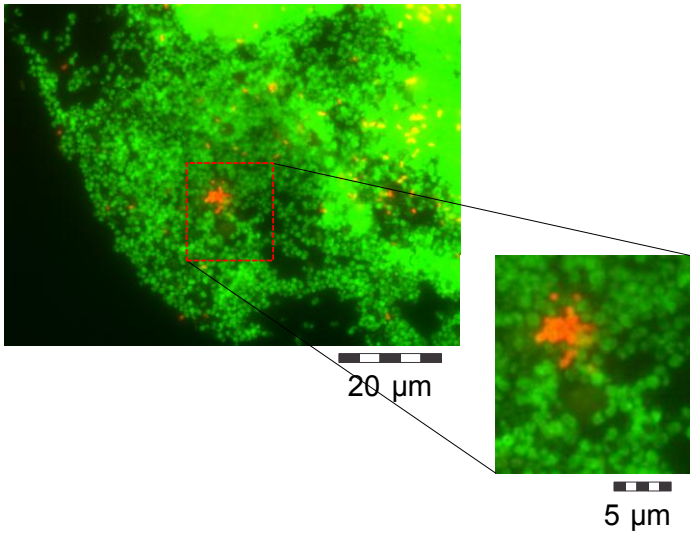


Figure 34: Overlap of FISH pictures and amplification. Green cells were only hybridized by EUBmix probe and the yellow/red cells represent the cells hybridized by Cf319a probe. Probes applied to sample I.

4.3.2.3. ANALYSIS AT *GENUS* LEVEL

After analyzing the evolution and composition of the culture, the next step was the refinement of the taxonomic composition of the *Alphaproteobacteria* community of the selected MMC at the end of the reactor operation. Based on some previous studies (Pisco *et al.* 2009), probes for *Sphingomonas*, *Defluvicoccus* and *Defluvicoccus* related Tetrad Forming Organism (TFO-DF) were applied, obtained only a positive result for TFO-DF (9.0 ± 0.28 %), as shown in the Figure 35. Besides the identification, it is possible to visualize clear PHA granules in the amplification.

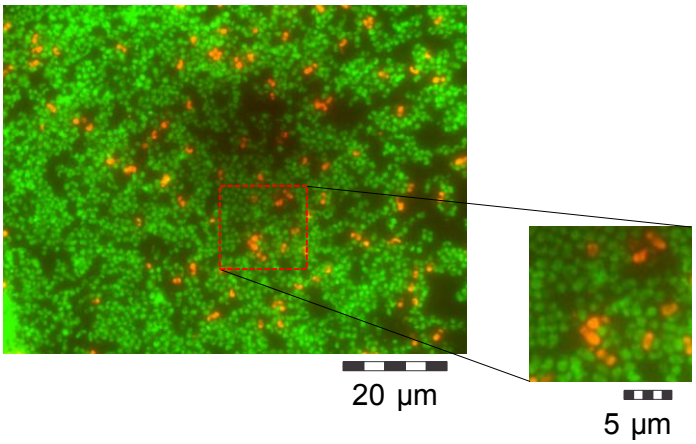


Figure 35: Overlap of FISH pictures and amplification. Green cells were only hybridized by EUBmix probe and the yellow/red cells represent the cells hybridized by TFO-DF 218+618 probe. Probes applied to sample I.

Still related with *Alphaproteobacteria* community, probes to identify bacteria belonging to genera *Paracoccus* and *Amaricoccus* were applied. The only positive result, and in a low concentration, was obtained for *Paracoccus*, accounting for only 4.2 ± 0.51 % of the *Ahphaproteobacteria* community, Figure 36. These probes were used once these genera were already identified as PHA-storing organisms in MMCs processes (Lemos *et al.* 2008).

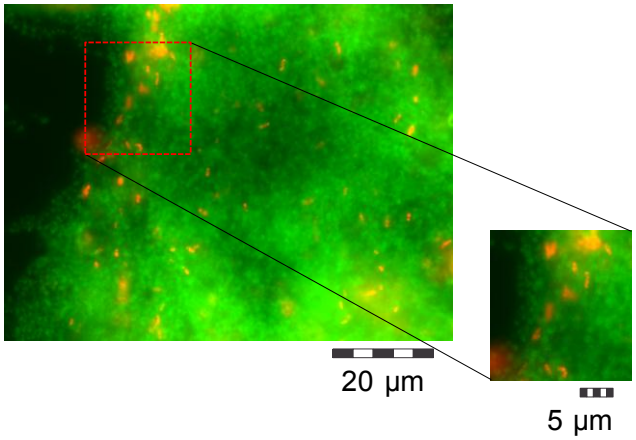
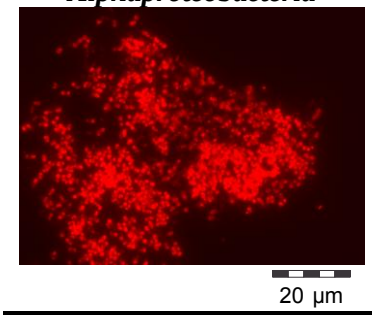
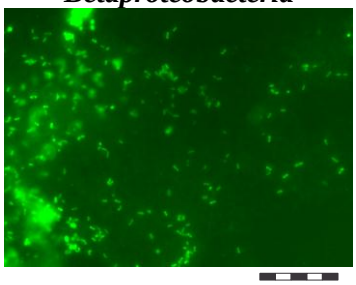
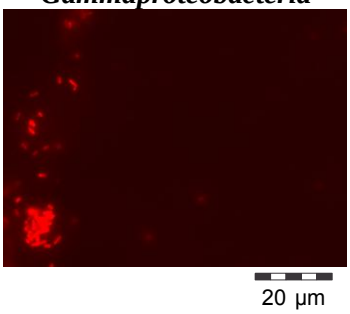


Figure 36: Overlap of FISH pictures and amplification. Green cells were only hybridized by EUBmix probe and the yellow/red cells represent the cells hybridized by Par651 probe. Probes applied to sample I.

Although the remaining populations, *Beta* and *Gammaproteobacteria*, exist in much lower concentrations when compared to *Alphaproteobacteria*, the existence of bacteria from the genera *Thauera* and *Azoarcus* (belonging to *Betaproteobacteria*) was also investigated due to their utilizations in bioremediations studies (Beristain-Cardoso *et al.* 2009; Farhadian *et al.* 2010), to the ability in using phenolic compounds by some species of this genera (Silva *et al.* 2010) and producing PHA (Lemos *et al.* 2008; Jiang *et al.* 2011a). This analysis was performed because was observed consumption of lignosulphonates from HSSL along the SBR cycle (Figure 18). However the results from FISH analysis were negative for both genera.

In order to a better understanding, Table 11 summarizes the principal results of the FISH and Staining analysis. Thereby, it is easier to establish a relation between the bacterial group/morphology/PHA accumulators storing.

Table 11: Summarize and relation of the main FISH and Staining results.

Bacterial Group	Bacterial Genera	Morphology	PHA accumulators
Alphaproteobacteria 	**Positive for:** *Paracoccus* *Defluvicoccus* - TFO **Negative for:** *Sphingomonas* *Amaricoccus* *Defluvicoccus*	**Main morphotypes**: *cocci, bacilli,* and some very cleary *cocobacilli*	✓ *Baccilli* ✓ *Cocci*
Betaproteobacteria 	Negative results for the probes applied: *Azoarcus* *Thauera*	**Main morphotypes**: *Bacilli*	✓ *Bacilli*
Gammaproteobacteria 	n/a	**Main morphotypes**: *Bacilli*	✓ *Bacilli*

4.3.3. ISOLATION ATTEMPTS

Due to the homogeneity of morphology presented by the selected MMC it was not possible to perform micromanipulation, and consequently the application of RT-PCR for a better identification of the selected microorganisms, an isolation approach was adopted. Biomass from the SBR collected on the tenth week was spread in solid medium containing MSV with acetic acid or xylose or the mixture of both and some colonies grew separately after 2 days. In this approach, only Gram negative bacteria presenting a *bacilli* morphology and belonging to *Gammaproteobacteria* were isolated, Figure 37. No bacteria belonging to *Alphaproteobacteria*, the dominant group on the selected MMC, or to *Betaproteobacteria* was isolated. Attempts in isolating organisms from activated sludge systems are quite frequent, however most of the times the target bacteria are only identified and their isolation reveals to be very difficult or even impossible. However Jiang *et al.* (2011) were able to isolate, after enriching a MMC in a SBR running under feast and famine conditions, a bacteria able to accumulate around 85 % of PHA also Gram negative and belonging to *Gammaproteobacteria*. The bacterium was identified as *Plasticicumulans acidivorans* (Jiang *et al.* 2011b).

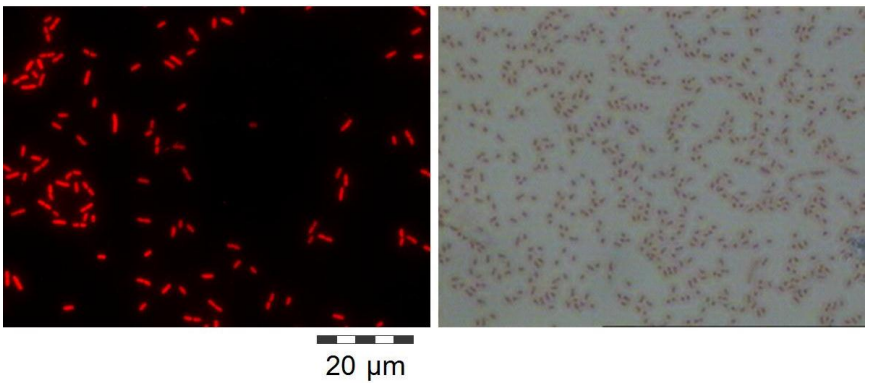


Figure 37: : FISH and Gram staining pictures of the pure culture isolated from the MMC. Red cells represent the bacteria belonging to *Gammaproteobacteria*, from plates with xylose as sole carbon source

After the isolation in Petri dishes, some of the isolated colonies were inoculated in liquid medium, MSV with acetic acid or xylose or the mixture of both as carbon source. Only in the medium containing xylose, cell growth was observed at a significant rate. No growth was observed on medium containing acetic acid or the mixture of xylose and acetic acid. This observation excluded the possibility of isolating *P. acidovorans* since it is described as able to grow in acetic acid and not in xylose (Jiang *et al.* 2011). After the adaption to the liquid medium, a kinetic test was performed, in

order to characterize the culture. Figure 38 presents the cellular growth and xylose consumption observed in this assay.

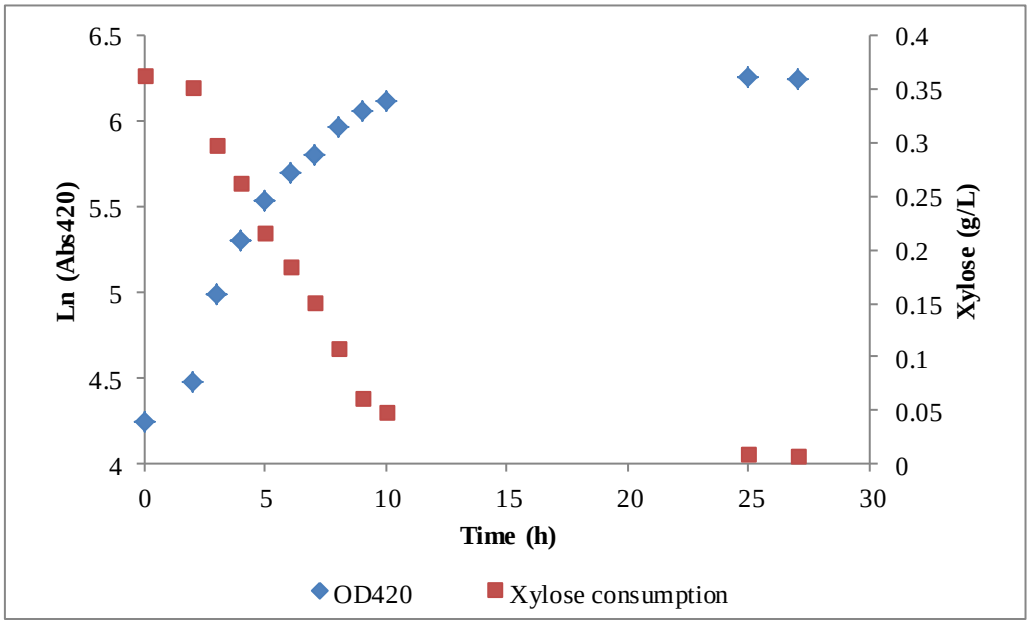


Figure 38: Microbial growth and xylose consumption evolution along the kinetic test performed with the isolated culture.

The isolated culture presented a maximum specific growth rate (μ_{max}) of 0.3 h^{-1} corresponding to duplication time of 2.3 h and a substrate uptake rate (r_s) of 0.036 gXyl/L.h. Phase contrast observation and Nile Blue staining were performed and the formation and increase of inclusion bodies during the growth was observed. An image of Nile blue staining of sample taken at time 5 h is shown in Figure 39.

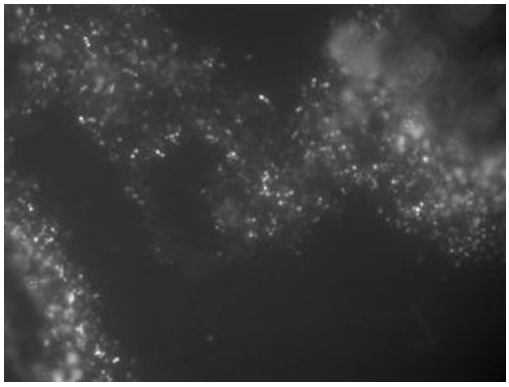


Figure 39: Nile Blue staining image at the end of the exponential phase of the pure culture, 5th hour.

Lopes and co-workers (2009) performed a screening of PHA-producing bacteria from xylose. The work started with a universe of 3152 isolated obtained from a

mangrove, a sewage treatment plant, the King George Island (Antarctic), the Amazon Rainforest, but only 164 of the isolates were able to grow in xylose and produce PHA. A small group of selected strains showed μ_{max} average of 0.37 h^{-1}, and an average lag phase of 2.5 h, and the OD600 nm reached to about 6.84 after 20 h of incubation (Lopes *et al.* 2009). These results are very similar to those obtained with the bacterial isolated from MMC.

The DNA of the isolated strain was extracted and PCR and Sequencing were performed, allowing for its identification as *Klebsiella spp*., which the preliminary affiliation was 99% to an uncultured *Klebsiella sp.* clone JXS1-28 (Peng *et al.* 2012). This organism is described as able to grow in xylose and to store PHA and is often found in activated sludge systems (Lopes *et al.* 2009; Razzaq *et al.* 2010). Wong *et al.* (2002) studied the production of PHA copolymers, from industrial waste, with different microorganisms isolated from activated sludge among which was *Klebsiella spp.* also from a mixed culture of activated sludge. Only the *Klebsiella spp.* was able to produced different copolymers, the ratios of HB:HV were 93:7 and 79:21 from malt waste and soy waste, respectively without co-substrate addition (Wong *et al.* 2002). Besides PHA, the *Klebsiella spp.* was already reported as capable to ferment the xylose to ethanol and 2,3-butanediol (Wu *et al.* 2008). From glycerol, *Klebsiella spp.* is also capable of producing 1,3-propanediol, a building-block for the chemical industry and can act as a precursor monomer along with acetic acid, in a proportion of 2:1for the synthesis of polyesters (Dworkin *et al.* 2006). A reduction in the pH medium to produce 1,3-propanediol, leads to the formation of 2,3-butanediol instead of acetic acid (Dworkin *et al.* 2006). However, some *Klebsiella* species are extremely pathogenic which could limit, for example, the use of PHA in medical applications (Dworkin *et al.* 2006). Further identification at the species level and pathogenesis degree should be determined in order to understand the possibility of using this microorganism in future studies regarding not only PHA production but also other value-added products from HSSL.

The successful attempt in isolating a PHA-storing microorganism using xylose seems a promising feature of this experimental work since only few xylose-utilizing bacteria are known. Nevertheless more isolation attempts using HSSL instead of pure xylose or acetic acid in solid medium should be performed. However this study should never put aside the use of MMCs for HSSL valorization. The need of substituting fossil raw materials by lignocellulosic ones requires all sort of biological processes to increase the amount of value-added products to obtain.

5. CONCLUSIONS

The operation of a SBR for 67 days under aerobic dynamic feeding with HSSL, a by-product of pulp industry, as a substrate allowed to select a MMC able to produce PHA. The MMC was able to successfully adapt to a carbon source quite diverse in its composition.

The operational conditions imposed resulted in a partial selection of the culture, since the xylose- and lignosulphonates-utilizing organisms never experienced a real famine phase but the obtained results showed that they were also involved in the PHA production. It is important to retain that, despite the culture was not fully selected, the maximum of PHA accumulation reached 67 %, a considerable value. Besides acetic acid, the other carbon sources present in HSSL should be looked with more attention because they could also represent a potential substrate for PHA production. However since a significant amount of xylose remaining at the end of the cycle indicates the possibility of integrating PHA process with another biological process where xylose is converted into a value-added product, namely bioethanol production by *S. stipitis.*

FISH analysis showed that the majority of the bacteria belonged to *Alphaproteobacteria*, which accounted to 72 % of the total bacteria. The remaining communities belonged to *Beta*- and *Gammaproteobacteria*, around 10 % each. The typical genera that are recognized to be selected in SBRs working under ADF conditions, *Amaricoccus, Azoarcus* and *Thauera,* were not detected.

Since it was not possible to perform micromanipulation, and consequently the application of RT-PCR, the isolation approach was adopted. This allowed to obtain an isolate, further identified as *Klebsiella spp.*. This genus was already reported as xylose-utilizing organism and able to produce some important precursors, PHA, bioethanol and also as recombinant organism.

With this project, the results obtained showed that it is possible to establish, not only, a process for the utilization of HSSL as substrate for production of PHA by a stable MMC within the biorefinery concept, but also to obtain new strains able to produce new chemicals.

6. FUTURE PROSPECTS

The study of using HSSL for PHA production is not finished with this work. The experiments that should followed this task include the study of several operational conditions in the selection SBR: substrate concentration and composition, organic loading rate (OLR), solids retention time, carbon to nitrogen ratio, pH, oxygen concentration, cycle length and feeding regimen. Also the role of other carbon compounds present in HSSL should be investigated since other sugars and lignosulphonates present can be consumed by the microorganisms. Also the role of the different compounds present in the HSSL (xylose, glucose, lignosulphonates) in microbial growth and PHA production should be evaluated. Moreover a detailed profile of the different compounds present in HSSL and secondary metabolites formed along the SBR cycle should be determined by GC-MS. In the same way, the polymer obtained should be well characterized in terms of its monomeric composition and properties.

Once the selection of a stable and well-characterized MMC, able to use the HSSL for PHA production, is obtained in the selection reactor, the next step is to start the optimization of PHA production by studying parameters as substrate concentration and composition, organic loading rate (OLR), solids retention time, carbon to nitrogen ratio, pH and oxygen concentration.

The possibility of using the remaining xylose, at the end of the cycle as substrate for other biological processes that use this pentose as carbon source should also be tested, namely bioethanol production from xylose by *Scheffersonmyces stipitis*. Moreover the moment when the effluent of the SBR should be collected and configuration of the selection reactor should be studied in order to maximize the availability of xylose. Furthermore, the possibility of using PHA production as a preliminary detoxification step should be assessed. As a result it is expected that the integration of PHA production with bioethanol fermentation in a HSSL-based biorefinery.

The characterization of the microbial community should continue since the dominant bacteria were only identified at class level. Additionally the presence and relative amount of the isolated strain in the selected MMC should be accounted by FISH analysis using the corresponding probe. The isolation attempts should continue but this time using HSSL as substrate. All the isolated strains must be characterised and their

ability in using the different carbon sources for growth and PHA production as well the type of polymer produced should be determined.

7. References

Akaraonye, E., T. Keshavarz and I. Roy (2010). "*Production of polyhydroxyalkanoates: the future green materials of choice*." Journal of Chemical Technology & Biotechnology **85**(6): 732-743.

Akiyama, M., T. Tsuge and Y. Doi (2003). "*Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation*." Polymer Degradation and Stability **80**(1): 183-194.

Albuquerque, M., M. Eiroa, C. Torres, B. Nunes and M. Reis (2007). "*Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses*." Journal of Biotechnology **130**(4): 411-421.

Albuquerque, M., C. Torres and M. Reis (2010). "*Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: Effect of the influent substrate concentration on culture selection*." Water Research **44**(11): 3419-3433.

Amann, R., B. Binder, R. Olson, S. Chisholm, R. Devereux and D. Stahl (1990). "*Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations*." Applied Environmental Microbiology **56**: 1919-1925.

Amann, R., W. Ludwig and K. Schleifer (1995). "*Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation*." Microbiological Reviews **59**(1): 143–169.

Anderson, A. and E. Dawes (1990). "*Occurence, metabolism, metabolic rate, and industrial uses of bacterial polyhydroxyalkanoates*." Microbiology Reviews **54**(4): 450-472.

Arcos-Hernandez, M., N. Gurieff, S. Pratt, P. Magnusson, A. Werker, A. Vargas and P. Lant (2010). "*Rapid quantification of intracellular PHA using infrared spectroscopy: An application in mixed cultures*." Journal of Biotechnology **150**(3): 372-379.

Atwood, J. and B. Rehm (2009). "*Protein engineering towards biotechnological production of bifunctional polyester beads*." Biotechnology Letters **1**(31): 131-137.

Beer, M., Y. Kong and R. Seviour (2004). "*Are some putative glycogen accumulating organisms (GAO) in anaerobic : aerobic activated sludge systems members of the -Proteobacteria?*" Microbiology **150**(7): 2267-2275.

Bengtsson, S., A. Werker, M. Christensson and T. Welander (2008). "*Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater*." Bioresource Technology **99**(3): 509-516.

Beristain-Cardoso, R., A. Texier, A. Alpuche-Solís, J. Gómez and E. Razo-Flores (2009). "*Phenol and sulfide oxidation in a denitrifying biofilm reactor and its microbial community analysis*." Process Biochemistry **44**(1): 23-28.

Bioplastics (2011). *What differentiates bioplastics from conventional plastics?, Retrieved 19.11.2011, http://en.european-bioplastics.org/bioplastics/.*

Carta, F., J. Beun, M. Van Loosdrecht and J. Heijnen (2001). "*Simultaneous storage and degradation of PHB and glycogen in activated sludge cultures*." Water Research **35**(11): 2693-2701.

Carvalheiro, F., L. Duarte and F. Gírio (2008). "*Hemicellulose biorefineries: a review on biomass pretreatments*." Journal of Scientific & Industrial Research **67**: 849-864.

Castilho, L., D. Mitchell and D. Freire (2009). "*Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation*." Bioresource Technology **100**(23): 5996-6009.

Chanprateep, S. (2010). "*Current trends in biodegradable polyhydroxyalkanoates*." Journal of Bioscience and Bioengineering **110**(6): 621-632.

Chen, G. (2009). "*A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry*." Chemical Society Reviews **38**(8): 2434-2446.

Choi, G., H. HKim and Y. Rhee (2004). "*Enzymatic and Non-enzymatic Degradation of Poly (3-Hydroxybutyrate-co-3-Hydroxyvalerate) Copolyesters Produced by Alcaligenes sp. MT-16*." The Journal of Microbiology **42**(4): 346-352.

Choi, J. and S. Lee (1997). "*Process analysis and economic evaluation for poly (3-hydroxybutyrate) production by fermentation*." Bioprocess Engineering **17**: 335-342.

Coats, E., F. Loge, M. Wolcott, K. Englund and A. McDonald (2007). "*Synthesis of Polyhydroxyalkanoates in Municipal Wastewater Treatment*." Water Environment Research **79**(12): 2396-2403.

Coats, E., K. VandeVoort, J. Darby and F. Loge (2011). "*Toward Polyhydroxyalkanoate Production Concurrent with Municipal Wastewater Treatment in a Sequencing Batch Reactor System*." Journal of Environmental Engineering **137**(1): 46-54.

Dai, Y., Z. Yuan, K. Jack and J. Keller (2007). "*Production of targeted poly(3-hydroxyalkanoates) copolymers by glycogen accumulating organisms using acetate as sole carbon source*." Journal of Biotechnology **129**: 489-497.

Dawes, E. and P. Senior (1973). "*The role and regulation of energy reserve polymers in microorganisms*." Advances in Microbial Physiology **10**: 135 - 266.

Dias, J., P. Lemos, L. Serafim, C. Oliveira, M. Eiroa, M. Albuquerque, A. Ramos, R. Oliveira and M. Reis (2006). "*Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product*." Macromolecular Bioscience **6**(11): 885-906.

Dionisi, D., M. Beccari, S. Di Gregorio, M. Majone, M. Papini and G. Vallini (2005). "*Storage of biodegradable polymers by an enriched microbial community in a sequencing batch reactor operated at high organic load rate*." Journal of Chemical Technology & Biotechnology **80**(11): 1306-1318.

Dionisi, D., G. Carucci, M. P. Papini, C. Riccardi, M. Majone and F. Carrasco (2005). "*Olive oil mill effluents as a feedstock for production of biodegradable polymers*." Water Research **39**(10): 2076-2084.

Dionisi, D., M. Majone, G. Vallini, S. Di Gregorio and M. Beccari (2006). "*Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor*." Biotechnology and Bioengineering **93**(1): 76-88.

Dircks, k., P. Pind, H. Mosbæk and M. Henze (1999). "*Yield determination by respirometry - The possible influence of storage under aerobic conditions in activated sludge*." Water SA **25**(1): 69-74.

Dworkin, M., S. Falkow, E. Rosenberg, K. Schleifer and E. Stackebrandt (2006). *The Prokaryotes. A handbook on the Biology of Bacteria: Proteobacteria: Gamma Subclass*, Springer.

Farhadian, M., D. Duchez, G. Gaudet and C. Larroche (2010). "*Biodegradation of toluene at high initial concentration in an organic–aqueous phase bioprocess with nitrate respiration*." Process Biochemistry **45**(11): 1758-1762.

Fuller, R. (1999). "*Microbial inclusions with special reference to PHA inclusions and intracellular boundary envelopes*." International Journal of Biological Macromolecules **25**: 21-29.

Ghosh, R. (2006). *Principles of Bioseparations Engineering* World Scientific Publishing Co. Pte. Ltd.

Gich, F., J. Garcia-Gil and J. Overmann (2001). "*Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes*." Archives of Microbiology **177**: 1-10.

Gironi, F. and V. Piemonte (2011). "*Bioplastics and Petroleum-based Plastics: Strengths and Weaknesses*." Energy Sources, Part A: Recovery, Utilization, and Environmental Effects **33**(21): 1949-1959.

Glazer, A. and H. Nikaido (2007). *Microbial Biotechnology: Fundamentals of Applied Microbiology*, CAMBRIDGE UNIVERSITY PRESS.

Gross, R. and B. Kalra (2002). "*Biodegradable Polymers for the Environment*." Science Magazine **297**(5582): 803-807.

Gurieff, N. and P. Lant (2007). "*Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production*." Bioresource Technology **98**(17): 3393-3403.

Hai, T., D. Lange, R. Rabus and A. Steinbuchel (2004). "*Polyhydroxyalkanoate (PHA) Accumulation in Sulfate-Reducing Bacteria and Identification of a Class III PHA Synthase (PhaEC) in Desulfococcus multivorans*." Applied and Environmental Microbiology **70**(8): 4440-4448.

Hess, A., B. Zarda, D. Hahn, A. Haner, D. Stax, P. Hohener and J. Zeyer (1997). "*In Situ Analysis of Denitrifying Toluene- and m-Xylene-Degrading Bacteria in a Diesel Fuel-Contaminated Laboratory Aquifer Column*." Applied and Environmental Microbiology **63**(6): 2136–2141.

Holmes, P. (1985). "*Application of PHB: a microbially produced biodegradable thermoplastics* " Physics in Technology **16**(1).

Hugenholz, P., G. Tyson, R. Webb, A. Wagner and L. Blackall (2000). "*Investigation of candidate division TM7, a recently recognized major lineage of the domain bacteria with no known pure-culture representatives*." Applied Environmental Microbiology(67): 411-419.

Jendrossek, D. and R. Handrick (2002). "*Microbial degradation of polyhydroxyalkanoates*." Annual Review of Microbiology **56**(1): 403-432.

Jenkins, D., M. Richard and G. Daigger (1986). *Manual on the causes and control of activated sludge bulking and forming.*, Water Research Comission.

Jiang, Y., L. Marang, R. Kleerebezem, G. Muyzer and M. van Loosdrecht (2010). "*Effect of temperature and cycle length on microbial competition in PHB-producing sequencing batch reactor*." The ISME Journal **5**(5): 896-907.

Jiang, Y., L. Marang, R. Kleerebezem, G. Muyzer and M. van Loosdrecht (2011a). "*Polyhydroxybutyrate production from lactate using a mixed microbial culture*." Biotechnology and Bioengineering **108**(9): 2022-2035.

Jiang, Y., D. Yu, R. Kleerebezem, G. Muyzer and M. Loosdrecht (2011b). "*Plasticicumulans acidivorans gen. nov., sp. nov., a polyhydroxyalkanoate-accumulating gammaproteobacterium from a sequencing-batch bioreactor*." International Journal of Systemtic and Evolutionary Microbiology **61**: 2314-2319.

Johnson, K., Y. Jiang, R. Kleerebezem, G. Muyzer and M. van Loosdrecht (2009). "*Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity*." Biomacromolecules **10**(4): 670-676.

Johnson, K., J. van Geest, R. Kleerebezem and M. van Loosdrecht (2010). "*Short- and long-term temperature effects on aerobic polyhydroxybutyrate producing mixed cultures*." Water Research **44**(6): 1689-1700.

Kadouri, D., E. Jurkevitch and Y. Okon (2003). "*Involvement of the Reserve Material Poly-β-Hydroxybutyrate in Azospirillum brasilense Stress Endurance and Root Colonization*." Applied Environmental Microbiology **69**(6): 3244–3250.

Kadouri, D., E. Jurkevitch and Y. Okon (2005). "*Ecological and Agricultural Significance of Bacterial Polyhydroxyalkanoates*." Critical Reviews in Microbiology **31**: 55-67.

Kamm, B., P. Gruber and M. Kamm (2006). *Biorrefinaries – Industrial Processes and Products.*, WILEY-VCH Verlag GmbH.

Karr, D., J. Waters and D. Emerich (1983). "*Analysis of Poly-,3-Hydroxybutyrate in Rhizobium japonicum Bacteroids by Ion-Exclusion High-Pressure Liquid Chromatography and UV Detection*." Applied and Environmental Microbiology **46**(6): 1339-1344.

Khanna, S. and A. Srivastava (2005). "*Recent advances in microbial polyhydroxyalkanoates*." Process Biochemistry **40**(2): 607-619.

Koller, M., I. Gasser, F. Schmid and G. Berg (2011). "*Linking ecology with economy: Insights into polyhydroxyalkanoate-producing microorganisms*." Engineering in Life Sciences **11**(3): 222-237.

Krishna, C. and M. Van Loosdrecht (1999). "*Effect of temperature on storage polymers and settleability of activated sludge*." Water Research **33**(10): 2374-2382.

Lajoie, C., A. Layton, I. Gregory, G. Sayler, D. Taylor and A. Meyers (2000). "*Zoogleal Clusters and Sludge Dewatering Potential in an Industrial Activated-Sludge Wastewater Treatment Plant*." Water Environment Research **72**(2): 56-64.

Lemoigne, M. (1926). "*Produit de deshydratation et de polymerisation de l'acide b-oxybutyrique*." Bull Soc Chim Biol **8**: 770-782.

Lemos, P., Y. Dai, Z. Yuan, H. Santos, J. Keller and M. Reis (2007). "*Metabolism of glycogen-accumulating organisms (GAO) revealed by in vivo 13C nuclear magnetic resonance*." Environemental Microbiology **9**: 2694-2706.

Lemos, P., S. L. and R. M. (2006). "*Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding*." Journal of Biotechenology **122**: 226-238.

Lemos, P., Levantesi, L. Serafim, S. Rossetti, M. Reis and V. Tandoi (2008). "*Microbial characterisation of polyhydroxyalkanoates storing populations selected under different operating conditions using a cell-sorting RT-PCR approach*." Applied Microbiology Biotechnology **78**: 351-360.

Lemos, P., L. Serafim and M. Reis (2006). "*Synthesis of polyhydroxyalkanoates from different short-chain fatty acids by mixed cultures submitted to aerobic dynamic feeding*." Journal of Biotechnology **122**(2): 226-238.

Liu, H., P. Hall, J. Darby, E. Coats, P. Green, D. Thompson and F. Loge (2008). "*Production of Polyhydroxyalkanoate During Treatment of Tomato Cannery Wastewater*." Water Environment Research **80**(4): 367-372.

Liu, Z., Y. Wang, N. He, J. Huang, K. Zhu, W. Shao, H. Wang, W. Yuan and Q. Li (2011). "*Optimization of polyhydrozybutyrate (PHB) production by excess activated sludge and microbial community analysis.* ." Journal of Hazardous Materials **185**(8-16).

Lopes, M., R. Rocha, S. Zanotto, J. Gomez and L. Silva (2009). "*Screening of bacteria to produce polyhydroxyalkanoates from xylose*." World Journal of Microbiology and Biotechnology **25**: 1751-1756.

Loy, A., A. Lehner, N. Lee, J. Adamczyk, H. Meier, J. Ernst, K. Schleifer and M. Wagner (2002). "*Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment*." Applied Environmental Microbiology **68**: 5064-5081.

Lücker, S., D. Steger, K. Kjeldsen, B. MacGregor, M. Wagner and A. Loy (2007). "*Improved 16S rRNA-targeted probe set for analysis of sulfate-reducing bacteria by fluorescence in situ hybridization*." Journal of Microbiological Methods **69**: 523-528.

Luengo, J., B. García, A. Sandoval, G. Naharro and E. Olivera (2003). "*Bioplastics from microorganisms*." Current Opinion in Microbiology **6**(3): 251-260.

Majone, M., P. Massanisso, A. Carucci, K. Lindrea and V. Tandoi (1996). "*Influence of storage on kinetic selection to control aerobic filamentous bulking*." Water Science and Technology **34**(5-6): 223-232.

Manz, W., R. Amann, W. Ludwig, M. Vancanneyt and K. Schleifer (1996). "*Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment*." Microbiology **142**: 1097-1106.

Manz, W., R. Amann, W. Ludwig, M. Wagner and K. Schleifer (1992). "*Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions*." Systematic and Applied Microbiology **15**: 593 - 600.

Maszenan, A., R. Seviour, B. Patel and J. Wanner (2000). "*A fluorescently-labelled r-RNA targeted oligonucleotide probe for the in situ detection of G-bacteria of the genus Amaricoccus in activated sludge* " Journal of Applied Microbiology **88**: 826-835.

Mccool, G. and M. Cannon (1999). "*Polyhydroxyalkanoate Inclusion Body-Associated Proteins and Coding Region in Bacillus megaterium*." Journal of Bacteriology **181**(2): 585-592.

Meier, H., R. Amann, W. Ludwig and S. K. (1999). "*Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content*." Systematic and Applied Microbiology **22**: 186-196.

Meyer, R., A. Saunders and L. Blackall (2006). "*Putative glycogen-accumulating organisms belonging to the Alphaproteobacteria identified through rRNA-based stable isotope probing*." Microbiology **152**(2): 419-429.

Miller, G. (1959). "*Use of Dinitrosalicylic Acid Reagent for determination of reducing sugar.*" Analytical Chemistry **31**: 426-428.

Moita, R. and P. Lemos (2011). "*Biopolymers production from mixed cultures and pyrolysis by-products*." Journal of Biotechnology **157**(4): 578-583.

Morgan-Sagastume, F., A. Karlsson, P. Johansson, S. Pratt, N. Boon, P. Lant and A. Werker (2010). "*Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus*." Water Research **44**(18): 5196-5211.

Muralikrishna, G., S. Schwarz, G. Dobleit, H. Fuhrmann and M. Krueger (2011). "*Fermentation of feruloyl and non-feruloyl xylooligosaccharides by mixed fecal cultures of human and cow: a comparative study in vitro*." European Food Research and Technology **232**(4): 601-611.

Neef, A. (1997). *Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen*, Technische Universität München. **PhD Thesis**.

Neef, A., R. Amann, H. Schlesner and K. Schleifer (1998). "*Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes*." Microbiology **144**: 3257-3266.

Neef, A., A. Zaglauer, H. Meier, R. Amann, H. Lemmer and K. Scheifer (1996). "*Population Analysis in a Denitrifying Sand Filter: Conventional and In Situ Identification of Paracoccus spp. in Methanol-Fed Biofilms.*" Applied and Environmental Microbiology **62**(12): 4329–4339.

Ojumu, T., J. Yu and B. Solomon (2004). "*Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer.*" African Journal of Biotechnology **3**(1): 18-24.

Peng, X., Z. Zhang, Z. Zhao and X. Jia (2012). "*16S ribosomal DNA clone libraries to reveal bacterial diversity in anaerobic reactor-degraded tetrabromobisphenol A.*" Bioresource Technology **112**: 75–82.

Penlogloua, G., C. Chatzidoukasa and C. Kiparissides (2011). "*Microbial production of polyhydroxybutyrate with tailor-made properties: An integrated modelling approach and experimental validation.*" Biotechnology Advances.

Pereira, S., S. Ivanuša, D. Evtuguin, L. Serafim and A. Xavier (2012). "*Biological treatment of eucalypt spent sulphite liquors: A way to boost the production of second generation bioethanol*." Bioresource Technology **103**(1): 131-135.

Philip, S., T. Keshavarz and I. Roy (2007). "*Polyhydroxyalkanoates: biodegradable polymers with a range of applications*." Journal of Chemical Technology & Biotechnology **82**(3): 233-247.

Pisco, A., S. Bengtsson, A. Werker, M. Reis and P. Lemos (2009). "*Community Structure Evolution and Enrichment of Glycogen-Accumulating Organisms Producing Polyhydroxyalkanoates from Fermented Molasses*." Applied and Environmental Microbiology **75**(14): 4676-4686.

Pontes, A. (2008). *Bioprocessameno de pentoses do licor de cozimento ao sulfito*. Chemistry Department. Aveiro, University of Aveiroo. **MSc. Thesis**.

Prior, B. and H. Potgieter (1981). "*Composition of fruit and vegetables processing wastes*." Water SA **7**(3): 155-159.

Queiroz, A. and F. Collares-Queiroz (2009). "*Innovation and industrial trends in bioplastics*." Polymer Reviews **49**(2): 65-78.

Razzaq, A., N. Jamil, N. Naheed and S. Hasnain (2010). "*Bacteria from contaminated urban and hilly areas as a source of polyhydroxyalkanoates production*." African Journal of Biotechnology **9**(13): 1919-1925.

Reddy, C., R. Ghai, Rashmi and V. Kalia (2003). "*Polyhydroxyalkanoates An overview*." Bioresource Technology **87**: 137-146.

Rees, G., G. Vasiliadis, J. May and R. Bayly (1992). "*Differentiation of polyphosphate and poly-b-hydroxybutyrate granules in an Acinetobacter sp. isolated from activated sludge*." FEMS Microbiology Letters **94**(1-2): 171-174.

Reis, M., L. Serafim, P. Lemos, A. Ramos, F. Aguiar and M. Van Loosdrecht (2003). "*Production of polyhydroxyalkanoates by mixed microbial cultures*." Bioprocess and Biosystems Engineering **25**(6): 377-385.

Restolho, J., A. Prates, M. de Pinho and M. Afonso (2009). "*Sugars and lignosulphonates recovery from eucalyptus spent sulphite liquor by membrane processes*." Biomass and Bioenergy **33**(11): 1558-1566.

Rodríguez-Carmona, E. and A. Villaverde (2010). "*Nanostructered bacterial materials for innovative medicines*." Trends in Microbiology **18**: 423-430.

Roller, C., M. Wagner, R. Amann, W. Ludwig and K. Schleifer (1994). "*In situ probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides*." Microbiology **140**: 2849-2858.

Salehizadeh, H. and M. Van Loosdrecht (2004). "*Production of polyhydroxyalkanoates by mixed cultures: recent trends and biotechnological importance*." Biotechnology Advances **22**: 261-279.

Satoh, H., Y. Iwamoto, T. Mino and T. Matsuo (1998). "*Activated Sludge as a possible source of biodegradable bioplastic*." Water Science & Technology **38**(2): 103-109.

Serafim, L., P. Lemos, M. Albuquerque and M. Reis (2008). "*Strategies for PHA production by mixed cultures and renewable waste materials*." Applied Microbiology and Biotechnology **81**(4): 615-628.

Serafim, L., P. Lemos, C. Levantesi, V. Tandoi, H. Santos and M. Reis (2002). "*Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms*." Journal of Microbiological Methods **51**: 1-18.

Serafim, L., P. Lemos, R. Oliveira and M. Reis (2004). "*Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions*." Biotechnology and Bioengineering **87**(2): 145-160.

Serafim, L., P. Lemos, S. Rossetti, C. Levantesi, V. Tandoi and M. Reis (2006). "*Microbial community analysis with a high PHA storage capacity*." Water Science & Technology **54**(1): 183.

Serafim, L. S., P. C. Lemos, R. Oliveira and M. A. M. Reis (2004). "*Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions*." Biotechnology and Bioengineering **87**(2): 145-160.

Serafim, L. S., P. C. Lemos, C. Torres, M. A. M. Reis and A. M. Ramos (2008). "*The Influence of Process Parameters on the Characteristics of Polyhydroxyalkanoates Produced by Mixed Cultures*." Macromolecular Bioscience **8**(4): 355-366.

Silva, C., A. Viero, A. Dias, F. Andreote, E. Jesus, S. De Paula, A. Torres, V. Santiago and V. Oliveira (2010). "*Monitoring the bacterial community dynamics in a petroleum refinery wastewater membrane bioreactor fed with a high phenolic load*." Journal of Microbiology and Biotechnology **20**(1): 21-29.

SIMRia (2011). *Sistema Municipal de Saneamento da Ria de Aveiro, Retrived 19.11.2011* *http://www.simria.pt/gca/?id=75&PHPSESSID=4bb38762aa2e388e0cb6d5376702c842*.

Singh, A. and N. Mallick (2008). "*Enhanced production of SCL-LCL-PHA co-polymer by sludge-isolated Pseudomonas aeruginosa MTCC 7925*." Letters in Applied Microbiology **46**(3): 350-357.

Sousa, L., S. Chundawat, V. Balan and B. Dale (2009). "*'Cradle-to-grave' assessment of existing lignocellulose pretreatment technologies*." Current Opinion in Biotechnology **20**(3): 339-347.

Stahl, D. and R. Amann (1991). *Development and application of nucleic acid probes.* Chichester, England, John Wiley & Sons Ltd.

Sudesh, K. and A. Doi (2000). "*Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters*." Progress in Polymer Science **25**: 1503-1555.

Sudesh, K. and T. Iwata (2008). "*Sustainability of Biobased and Biodegradable Plastics*." CLEAN - Soil, Air, Water **36**(5-6): 433-442.

Takabatake, H., H. Satoh, T. Mino and T. Matsuo (2000). "*Recovery of biodegradable plastics from activated sludge process*." Water Science and Technology **42**(3): 351-356.

Third, K., M. Newland and R. Cord-Ruwisch (2003). "*The effect of dissolved oxygen on PHB accumulation in activated sludge cultures*." Biotechnology and Bioengineering **82**(2): 238-250.

Tsuge, T. (2002). "*Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates.*" Journal of Bioscience and Bioengineering **94**(6): 579-584.

Verlinden, R., D. Hill, M. Kenward, C. Williams and I. Radecka (2007). "*Bacterial synthesis of biodegradable polyhydroxyalkanoates*." Journal of Applied Microbiology **102**: 1437–1449.

Villano, M., M. Beccari, D. Dionisi, S. Lampis, A. Miccheli, G. Vallini and M. Majone (2010). "*Effect of pH on the production of bacterial polyhydroxyalkanoates by mixed cultures enriched under periodic feeding*." Process Biochemistry **45**(5): 714-723.

Wallen, L. and W. Rohwedder (1974). "*Poly-b-hydroxyalkanoate from activated sludge*." Environmental Science Technology **8**: 576-579.

Wong, M., F. Tan, W. Ng and W. Liu (2004). "*Identification and occurrence of tetrad-forming Alphaproteobacteria in anaerobic-aerobic activated sludge processes*." Microbiology **150**(11): 3741-3748.

Wong, P., H. Chua, W. Lo, H. Lawford and P. Yu (2002). "*Production of Specific Copolymers of Polyhydroxyalkanoates from Industrial Waste*." Applied Biochemistry and Biotechnology **98**: 665-662.

Wu, K., G. Saratale, Y. Lo, W. Chen, Z. Tseng, M. Chang, B. Tsai, A. Su and J. Chang (2008). "*Simultaneous production of 2,3-butanediol, ethanol and hydrogen with a Klebsiella sp. strain isolated from sewage sludge*." Bioresource Technology **99**(17): 7966-7970.

Wu, Q., Y. Wang and G. Chen (2009). "*Medical application of microbial biopolyesters polyhydroxyalkanoates*." Artificial Cells, Blood Substitutes and Biotechnology **37**: 1-12.

Xavier, A., M. Correia, S. Pereira and D. Evtuguin (2010). "*Second-generation bioethanol from eucalypt sulphite spent liquor*." Bioresource Technology **101**(8): 2755-2761.

Yang, S. (2007). *Bioprocessing for value-added products from renewable resources: New technologies and applications.*, Elsevier Science & Technology Books.

Yao, Y., X. Zhan, J. Zhang, X. Zou, Z. Wang, Y. Xiong, J. Chen and G. Chen (2008). "*A specific drug targeting system based on polyhydroxyalkanoate granule binding protein PhaP fused with targeted cell ligands*." Biomaterials **29**: 4823-4830.

Yilmaz, M., H. Soran and Y. Beyatli (2005). "*Determination of poly-β-hydroxybutyrate (PHB) production by some Bacillus spp.*" World Journal of Microbiology and Biotechnology **21**(4): 565-566.